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Perinatal Cardiac Function in Congenital Diaphragmatic Hernia

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Ciências da Saúde – Ciências Biológicas e Biomédicas

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Declaração

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Perinatal Cardiac Function in Congenital Diaphragmatic Hernia

Função Cardíaca Perinatal na Hérnia Diafragmática Congénita

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‘Our future challenge remains to develop novel approaches in the treatment of infants with congenital diaphragmatic hernia, as well as the perfection of innovative strategies already existing through ongoing laboratory and clinical research.’

Michael S. Irish, 1996

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Gabriel Garcia Marqués diz que todos nascemos e morremos sozinhos. Pelo contrário, concretizar uma Tese de Doutoramento não é, definitivamente, um acto solitário. Como tal, expresso o meu agradecimento a todos aqueles que me apoiaram, motivaram e ensinaram durante os últimos anos. A rotina não é entediante para um médico que tenta manter uma actividade constante entre o serviço hospitalar, o trabalho de investigação e a actividade docente. Pelo caminho, vão-se perdendo sorrisos e alegrias que, no imediato, nem sempre nos afiguram recompensadas. Por isso, o apoio de tutores, colegas e amigos, nas suas diversas vertentes, são pedra angular para quem opta por trilhos menos fáceis. Na concretização desta Tese de Doutoramento várias têm sido as pessoas com papel relevante. No Instituto de Ciências da Vida e da Saúde, bem como no Serviço de Cardiologia Pediátrica, o espírito de colaboração foi uma constante. Em consequência, expresso o meu agradecimento:

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ABSTRACT

Congenital diaphragmatic hernia (CDH) is a congenital condition with variable illness severity that usually requires complex and multidisciplinary care. The physiologic consequences of this defect may be mild and minimally symptomatic at birth, but almost half of CDH infants present with severe forms of the disease, with poor outcome and extremely high mortality, despite aggressive treatment even in experienced teams with sophisticated management protocols. Morbidity and mortality associated with CDH is largely dependent of lung hypoplasia and pulmonary hypertension (PH). Most severe CDH newborns clinically resemble infants in terminal heart failure, even in the absence of cardiac malformation. During the last decade several authors suggested the existence of heart hypoplasia and immaturity in CDH, as occurs in lung. From our initial works we defined that heart hypoplasia in the experimental rat model of CDH was related with nitrofen and occurs only in early phases of gestation. In addition, in that model, prenatal treatment with vitamin A partially prevents the diaphragmatic defect and lung hypoplasia but only interfere with the molecular effect of nitrofen in lung hypoplasia. The absence of heart hypoplasia did not preclude the existence of some degree of molecular heart immatury that hampers heart function in pressure overload conditions. We exhaustively investigated throughout gestation several molecular parameters of heart development and we didn't find any molecular signs of myocardium immaturity in this experimental model of CDH, since the molecular parameters were similar in all studied groups (control, nitrofen and CDH groups). With these results, we exclude the hypothesis that heart hypoplasia or immature could be the *missing link* in CDH responsible for greater mortality. We hypothesize that heart failure in CDH infants might be caused essentially by cardiac overload secondary to severe PH. In the experimental model of nitrofen induced CDH we demonstrated genetic molecular expression of the cardiac overload markers B type natriuretic peptide, endothelin-1

and angiotensinogen throughout gestation and after birth. We found that although during gestation the cardiac expression of those markers was similar in CDH and control fetuses, after birth there are a significant increase in the expression of all studied markers in right ventricle (RV) in CDH group. Simultaneously, in a clinical study we demonstrated that CDH Human infants present echocardiographic evidence of heart function adaptation, systolic and diastolic, in both ventricles. The heart dysfunction was also demonstrated by the increase in plasmatic N-terminal-pro- B type natriuretic peptide (NT-proBNP) level, an overload biochemical marker. Moreover, plasmatic levels of NT-proBNP had an excellent correlation with PH and seem to have prognostic value in CDH. Taking into account all these findings, we suggest a protocol to evaluate PH and heart function in CDH infants, based in echocardiographic and serical parameters. This protocol would be useful to establish prognosis, follow-up of the clinical evolution, support the decision to pulmonary vasodilators therapy, and will help in the decision of the moment to surgical repair of CDH infants.

RESUMO

A Hérnia Diafragmática Congénita (HDC) é uma perturbação do desenvolvimento embrionário que pode afectar diferentes órgãos para além do diafragma e do pulmão, como o coração ou o rim, exigindo uma abordagem complexa e multidisciplinar. As consequências fisiológicas da doença podem ser variáveis, existindo formas muito leves e minimamente sintomáticas até quadros clínicos em que se associam múltiplas malformações incompatíveis com a vida. No entanto, cerca de metade dos recém-nascidos (RN) afectados apresentam-se com formas caracterizadas por hipoplasia pulmonar grave, com prognóstico reservado e mortalidade inaceitavelmente elevada, apesar da disponibilização de abordagens terapêuticas agressivas e sofisticadas, mesmo em centros de referência. Actualmente, considera-se que a morbilidade e a mortalidade associadas à HDC estão dependentes da gravidade da hipoplasia pulmonar e da hipertensão pulmonar (HTP) existentes. No entanto, a evolução dos RN afectados caracteriza-se por uma grande imprevisibilidade, com agravamentos clínicos sustentados que muitas vezes não respondem à terapêutica instituída. Da experiência acumulada no tratamento destes RN apercebemo-nos de que os doentes mais gravemente afectados apresentam uma instabilidade ventilatória e hemodinâmica semelhante à da insuficiência cardíaca terminal, mesmo na ausência de doença cardíaca estrutural. Durante a última década tem sido sugerida a existência de hipoplasia e/ou imaturidade cardíaca nestes doentes. Os estudos desenvolvidos pelo nosso grupo demonstraram que no modelo da HDC induzida experimentalmente pelo nitrofeno verifica-se hipoplasia cardíaca apenas causada pelo nitrofeno e em fases precoces do desenvolvimento fetal. Neste modelo demonstramos que o tratamento prenatal com vitamina A previne parcialmente a ocorrência de defeito diafragmático e melhora o crescimento pulmonar, interferindo apenas nos mecanismos moleculares relacionados com o nitrofeno. A inexistência de hipoplasia cardíaca no termo da gestação, não excluía algum grau de imaturidade que eventualmente

comprometesse a função cardíaca em situações de sobrecarga ventricular de pressão. Assim, realizámos um estudo exaustivo para avaliar a expressão de diversos parâmetros moleculares de desenvolvimento cardíaco, ao longo da gestação, e não encontrámos qualquer evidência molecular de imaturidade miocárdica, pelo que excluímos a hipótese de existir hipoplasia ou imaturidade cardíaca que contribuísse para a elevada mortalidade destes doentes, valorizando a importância da sobrecarga de pressão causada pela HTP na função ventricular. No modelo experimental da HDC, demonstrámos que a expressão génica de marcadores de sobrecarga ventricular (peptídeo natriurético do tipo B, endotelina 1 e angiotensinogénio) não sofria qualquer alteração durante a gestação, registando-se um significativo aumento da sua expressão ventricular direita após o nascimento no grupo HDC. Simultaneamente, num estudo clínico efectuado demonstrámos que RN Humanos com HDC apresentam evidência ecocardiográfica de adaptação da função cardíaca, sistólica e diastólica, em ambos os ventrículos. Esta disfunção foi também demonstrada pelo aumento do fragmento N terminal do peptideo natriurético tipo B (NT-proBNP), um marcador bioquímico de sobrecarga ventricular. Os níveis plasmáticos de NT-proBNP apresentam uma excelente correlação com a HTP e parecem ter interesse prognóstico nos RN com HDC. Com base no trabalho desenvolvido propomos um protocolo de avaliação da HTP e da função cardíaca baseado em parâmetros ecocardiográficos e bioquímicos que possam monitorizar a evolução clínica, fundamentar a decisão de inicio de terapêutica vasodilatadora pulmonar, decidir o momento ideal para a cirurgia de reparação bem como estabelecer o prognóstico dos RN com HDC.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme

ADMA, nitric oxide synthase inhibitor asymmetrical dimethylarginine

ASD, atrial septal defect

ATP, adenosine triphosphate

bHLH, basic-helix-loop-helix

BMP, bone morphogenic protein

BNP, B type natriuretic peptide

CDH, congenital diaphragmatic hernia

cGMP, cyclic guanosine monophosphate

c-met, cytoplasmatic domain of the hepatocyte growth factor receptor

COUP-FT2, chicken ovoalbumin upstream promoter-transcription factor

CTn, cardiac troponin

Dpc, days post conception

ECMO, extracorporeal circulation membrane oxygenation

EMAP II, endothelial monocyte activating polypeptide II

eNOS, endothelial nitric oxide synthase

ET, endothelin

FGF, fibroblast growth factor

FOG, friend of GATA

GATA, zinc-finger transcription factor

IGF, insulin-like growth factors

Irx4, iroquois homeobox gene

LOX, lecithin-like oxidized-low density lipoprotein receptor-1

LV, left ventricle

MLC, myosin light chain

NICU, neonatal intensive care unit

NO, nitric oxide

NT-proBNP, N-terminal-pro-B type natriuretic peptide

PAP, pulmonary artery pressure

PASP, pulmonary artery systolic pressure

PH, pulmonary hypertension

PPF, pleuroperitoneal fold

RA, retinoic acid

RALDH, retinaldehyde dehydrogenase

RAR, retinoic acid receptor

RV, right ventricle

RXR, retinoic acid receptor X

SERCA, sarcoplasmic reticulum calcium

Shh, sonic hedgehog

TDI, tissue Doppler imaging

TGF, transforming growth factor

TOF, tetralogy of Fallot

TR, tricuspid regurgitation

VEGF, vascular endothelial growth factor

VSD, ventricular septal defect

Wt1, Wilms' tumor 1 gene

CHAPTER I

GENERAL INTRODUCTION AND AIMS

1. CLINICAL RELEVANCE OF CONGENITAL DIAPHRAGMATIC HERNIA

Congenital diaphragmatic hernia (CDH) is a relatively infrequent condition (~1:2000 births) with variable illness severity that usually requires complex and multidisciplinary care. This illness has been described as a developmental insult, with a simple anatomic defect in which a hole in the diaphragm allows abdominal viscera to herniate into the thorax. The physiologic consequences of this defect may be mild and minimally symptomatic at birth, but often they are severe. In our days, CDH is seen as a complex syndrome with frequent association with cromossomic anomalies and malformations, mainly cardiac, renal and skeletal.

Almost half of CDH infants present with severe forms of the disease, predominantly with lung and diaphragmatic anomalies. These patients have poor outcome and extremely high mortality (~50%) despite aggressive treatment, even in experienced teams with sophisticated management protocols. Several authors believe that morbidity and mortality associated with CDH is largely dependent on lung hypoplasia and pulmonary hypertension (PH). Pulmonary hypoplasia installs during prenatal development and limited possibilities exist to attenuate it. PH is likely secondary to pulmonary hypoplasia and associated underdeveloped vascular bed, being for some authors the major determinant of postnatal clinical outcome.

Regardless of experimental and clinical efforts using new modalities directed toward reduction of PH and/or improved pulmonary gas exchange in CDH infants, there has been relatively little impact on survival of the most severely affected subset of CDH infants. Antenatal therapies that promote lung growth before birth have been looked for, as an appealing approach for fetuses with severe CDH. However, fetal surgical intervention is invasive, technically demanding, and limited by the maternal and fetal risks. Less invasive approaches such as antenatal pharmacological treatment or

gene therapy to stimulate lung growth and maturation is being also considered. However, any of the above strategies had resulted in significant clinical impact.

In our days, the postnatal therapy persists as the main approach in CDH. The diaphragmatic surgical repair is usually delayed until respiratory and hemodynamic stabilization and PH controlled. The medical management of these patients is designed to achieve an optimal oxygenation and is based in pulmonary vasodilators, high frequency ventilation, extracorporeal membrane oxygenation (ECMO) and surfactant administration. Nevertheless, the decision to start more aggressive therapy, like ECMO, and its monitorization, should be based in accurate parameters. In several centers, the evaluation of PH severity is considered the most important aspect in management of CDH infants. The assessment of pulmonary artery pressure is mainly based on clinical and echocardiographic estimation. However, echocardiographic evaluation is not always available and it is somewhat observer-dependent and technically demanding in CDH infants due to the presence of abdominal organs in thoracic cavity. An easy and reliable method to assess PH remains to be defined and the identification of reliable prognostic parameters that can orient the clinical strategies is therefore warranted.

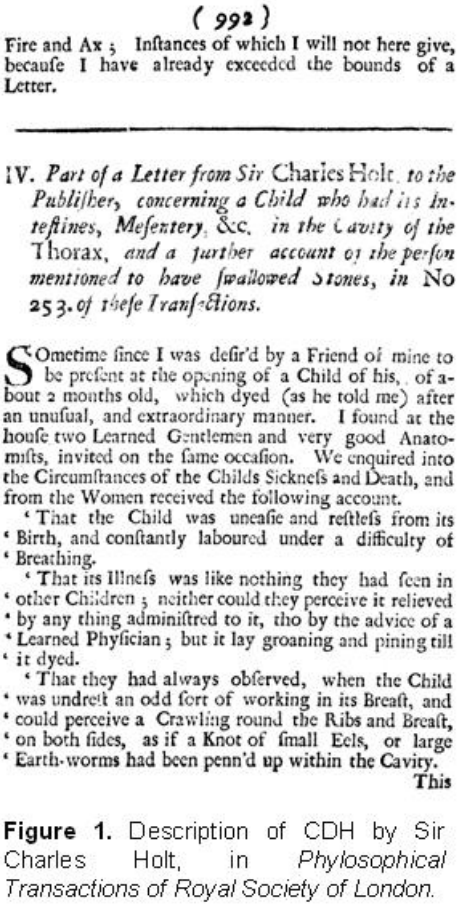
According to this background, CDH persists as one of the main challenge in perinatology. It is imperative understand the basic mechanisms of the disease, in order to achieve a better clinical approach of affected infants. This justifies widespread deep and exhaustive investigation in this particular field of health sciences. This thesis reflects the work from our group and our attempts to clarify the hemodynamic aspects that could interfere in the outcome of CDH infants.

2. HISTORICAL REVIEW

If men could learn from history, what lessons it might teach us!

Coleridge ST (1772-1834)

The earliest recordings of diaphragmatic hernia comes from Hippocrates (460-370 BC), indicated it being caused by traumatic perforations, usually with herniation of abdominal viscera into the thoracic cavity, and subsequent death [Coar, 1822]. Nevertheless, the first *congenital* diaphragmatic hernia was recorded only in 1679 by Lazarus Riverius, as an incidental postmortem finding in a 24-year-old man [Riverius, 1679]. The first pediatric account of CDH was recorded in 1701 by Sir Charles Holt, who describes the clinical and postmortem findings of a two months old infant with CDH (Figure 1) [Holt, 1701].



(992)

Fire and Ax; Inſtances of which I will not here give, becauſe I have already exceeded the bounds of a Letter.

IV. *Part of a Letter from Sir* Charles Holt, *to the Publiſher, concerning a Child who had its Inteſtines, Meſentery,* &c. *in the Cavity of the* Thorax, *and a further account of the perſon mentioned to have ſwallowed Stones, in* No 253. *of theſe Tranſactions.*

SOmetime ſince I was deſir'd by a Friend of mine to be preſent at the opening of a Child of his, of about 2 months old, which dyed (as he told me) after an unuſual, and extraordinary manner. I found at the houſe two Learned Gentlemen and very good Anatomiſts, invited on the ſame occaſion. We enquired into the Circumſtances of the Childs Sickneſs and Death, and from the Women received the following account.

'That the Child was uneaſie and reſtleſs from its 'Birth, and conſtantly laboured under a difficulty of 'Breathing.

'That its Illneſs was like nothing they had ſeen in 'other Children; neither could they perceive it relieved 'by any thing adminiſtred to it, tho by the advice of a 'Learned Phyſician; but it lay groaning and pining till 'it dyed.

'That they had always obſerved, when the Child 'was undreſt an odd ſort of working in its Breaſt, and 'could perceive a Crawling round the Ribs and Breaſt, 'on both ſides, as if a Knot of ſmall Eels, or large 'Earth-worms had been penn'd up within the Cavity.

This

Figure 1. Description of CDH by Sir Charles Holt, in *Phylosophical Transactions of Royal Society of London.*

In 1761, Morgagni described various types of diaphragmatic hernias, including the anterior diaphragmatic hernia which bears his name [Morgnani, 1769]. He further referred the small size of the ipsilateral lung, thus being the earliest account of pulmonary hypoplasia in association with CDH. In 1848, Bochdalek, whose name would became an eponym for CDH, described CDH occurring through both right and left posterolateral diaphragmatic defects [Bochkdalek, 1848].

In 1888 Neumanm attempt the first CDH correction on a 19-year-old patient [Naumann, 1888] and in 1898 O'Dwyer performed the first abdominal approach for repair of CDH in a 3-year-old infant [Pilcher *et al.,* 1890]. Both patients died. The first successful repair of CDH was reported only in 1902, by Heidenhain, in a 9 year-old-boy [Heidenhain, 1905]. In 1925, Hebdolm reported 75% mortality in 44 congenital cases reviewed and suggested that early surgery of CDH might improve survival [Hedbolm, 1925]. The first successful repair of CDH in an infant was reported by Bettman and Hess in 1929 [Bettman & Hess, 1929]. Nevertheless, only in 1946 Gross went on to report the first successful repair of a neonate less than 24 hours of age [Gross, 1946]. In contrast to this view of early operation, several authors have advocate and shown benefit to delayed surgical repair of CDH, after clinical stabilization.

In the meantime, in the sequence of embryonic development investigation, Broman, in 1902, conclude that the absence of pleuro-peritoneal closure could result in diaphragmatic defect. In 1921 Korns recognized the importance of pulmonary hypoplasia in CDH and in 1953 Campanale and Rowland produced the first important paper extensively documenting the severe ipsilateral pulmonary hypoplasia, as well as degree of contralateral hypoplasia in CDH. In 1970, Murdock and Rowe demonstrated the role of acid-base and blood gas analysis in the determination of prognosis and therapy in CDH [Irish *et al.*, 1996].

The introduction of the fetal lamb model of CDH by Lorimier and coworkers in 1967 provided a reliable means for studying the pathophysiology of CDH [de Lorimier *et al.*, 1967]. From 1990 to our days, the rat model of nitrofen induced CDH is the most used on experimental research due to easier manipulation and similarity with human disease [Tenbrick *et al.*, 1990; Kluth *et al.*, 1990]. Both models, which closely parallel the pathophysiologic consequences of CDH in humans, are used extensively to

advance our understanding of this condition. Furthermore, they had provided a mean of investigation and implementing several innovative strategies in the treatment of CDH.

As our understanding of pulmonary hypoplasia and PH has evolved, CDH has become a physiologic emergency rather than a surgical one. The realization that early surgical repair was associated with unfavorable changes in lung compliance and gas exchange, resulted in the rationale for a period of preoperative stabilization and delayed surgery [Nair *et al.*, 1983; Cartlidge *et al.*, 1986; Charlton *et al.*, 1991]. In our days, initial therapy is directed toward hemodynamic stabilization and respiratory support. This awareness results in improvement in therapeutics, like pulmonary vasodilators, sophisticated ventilatory strategies and ECMO [Nagaya *et al.*, 1991; Reyes *et al.*, 1998; Bartlett, 2005]. Prenatal diagnosis is possible from routine obstetric screening from 15 week's gestation and several studies are currently evaluating the benefit of prenatal therapy, namely *in utero* surgery or genetic modulation of lung growth [Henriques-Coelho *et al.*, 2004; Santos *et al.*, 2006].

3. Normal and CDH Abnormal Embryonic and Fetal Development

In the next sections, development of diaphragm, lung, heart and pulmonary vasculature will be briefly reviewed and focused in pathophysiologic aspects of CDH.

3.1 Diaphragm development

In Humans, the separation of the lateral plate of the intra-embryonic mesoderm occurs at the end of third week of gestation. This will determine the establishment of coelomic cavity, extending from the thoracic to the pelvic region. The most important structure

dividing the intra-embryonic coelomic cavity is formed by the septum transversum, a thick plate of mesodermal tissue occupying the space between the thoracic cavity and the stalk of the yolk sac (Figure 2). This septum does not separate the thoracic and abdominal cavities entirely, but leaves a large opening, the pericardioperitoneal canal, on each side of the foregut.

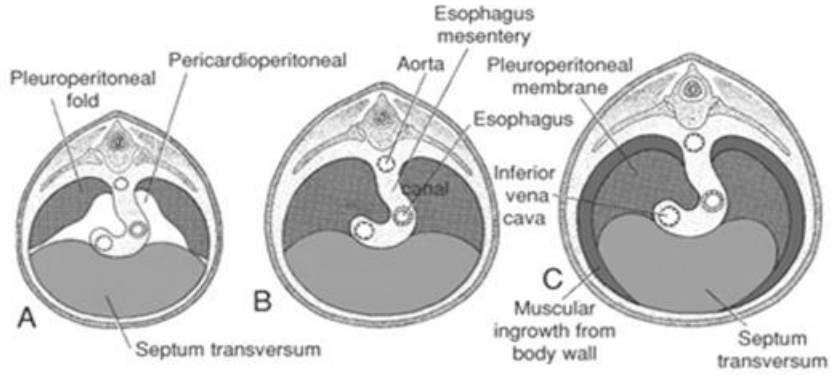


Figure 2. Schematic drawings illustrating the development of the diaphragm. (A) The pleuroperitoneal folds appear at 5th week. (B) The pleuroperitoneal folds have fused with the septum transversum and the mesentery of the esophagus in the 7th week, thus separating the thoracic cavity from abdominal cavity. (C) Transverse section at the 4th month of development.

According to the classical texts of embryology, the diaphragm derived from four distinct embryologic structures: i) the septum transversum, which forms the tendinous part of the diaphragm, ii) the two pleuroperitoneal membranes, iii) muscular components from the lateral and dorsal body walls, and iv) the mesentery of the esophagus, in which the crura of the diaphragm develop. Hence, diaphragmatic closure, determining the definite separation of the thoracic and abdominal portions of the coelomic cavity is determined by the pleuroperitoneal membrane, at the eight week's gestation [wells, 1954].

Examination of mouse models didn't supported the hypothesis that diaphragmatic musculature is derived from the esophageal mesentery or ingrowth of muscle from the lateral body wall. The contribution of mesoderm to different regions of the

diaphragm seems to be essential, but not clearly determined. The somatic mesoderm may originate the pleuroperitoneal fold (PPF), a structure that will form the posterior diaphragm [Babiuk *et al.*, 2003]. A sub-type of the somatic mesoderm, the septum transversum mesoderm, contributes to the anterior diaphragm [Babiuk *et al.*, 2003; Liu *et al.*, 2003; Yuan *et al.*, 2003]. The PPF is a pyramid-shaped tissue that extends medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum (a distinct structure that will form the central tendon of the diaphragm).

Several authors suggest that the embryogenesis of the PPF parallels that of the forelimb bud. Specifically, the substructure of the PPF is derived from mesenchymal cells migrating from the somatic mesoderm. Subsequently, muscle precursors migrating from the dermomyotome of cervical somites follow the guidance cues provided by the somatopleure substructure and become localized in the PPF. Afterwards, muscle and neuronal precursors migrate from the 3º, 4º and 5º cervical somites through PPF, leading to the formation of the muscular and neuronal components of the diaphragm, at the 4ª week's gestation [Sze *et al*., 1995; Babiuk *et al.*, 2003]. Growth and development of adjacent cells is probably dependent on growth factors secreted by the distal ends of PPF, but these mechanisms are still not completely understood.

Additionally, the significance of mesenchyme in the formation of the membranous portion of the diaphragm has been re-enforced [Yuan *et al.*, 2003]. Mice lacking the gene *Slit3* (-/-) develop a central (septum transversum) CDH. *Slit3* encodes a member of the *Slit* family of guidance molecules and is expressed predominantly in the mesothelium of the diaphragm during embryonic development. In *Slit3* null mice, the central tendon region of the diaphragm fails to separate from liver tissue because

of abnormalities in morphogenesis. The CDH progresses through continuous growth of the liver into the thoracic cavity.

While the mechanisms underlying the etiology of CDH remain obscure, several theories pertaining to the pathogenesis of the condition have been proposed. The most commonly cited explanation for CDH states that there is an abnormality with the closure of the pleuroperitoneal canal. However, contrary to the often-documented theory, several data from animal models of CDH do not support this hypothesis. First, the diaphragm defects usually arise medially of the pleuroperitoneal canals, and the two can be spatially separated in these diaphragms. Secondly, nitrofen causes the formation of well-defined holes in the diaphragm which are clearly evident on either the right or left side at 14.5 days postconception (dpc), approximately 0.25 and 0.75 days prior to the closure of the right and left pleuroperitoneal canals, respectively.

One inquisitive aspect in the CDH patophysiology is the presence of bilateral pulmonary hypoplasia from early stages of lung development. Based on this observation several authors suggested that the diaphragmatic defect in CDH could be secondary to lung hypoplasia, due to the absence of critical signals emanating from adjacent lung. Nevertheless, further studies established that the diaphragmatic defect is independent, as well as previous or simultaneous to the pulmonary hypoplasia. In fact, in *Fgf10*(-/-) null mutant mice, that do not develop lung tissue, Greer *et al* demonstrated that the diaphragm forms normally in the absence of any putative associated growth-related signals [Babiuk *et al.,* 2002]. Thus the diaphragmatic defects associated with CDH are a primary defect and not a secondary result of lung malformation. Convincing evidence suggest that there being a common mechanism underlying the pathogenesis of CDH that targets primordial diaphragm and lung development in parallel.

One of the most consistent hypothesis supports that defects can be traced back to much earlier stages of development during the formation of the primordial diaphragm and could be related to a defective PPF. Three-dimensional reconstructions of the PPF have demonstrated that the malformed areas in the animal model of CDH are consistently located in the dorsolateral region, the area affected in human CDH. The mesenchymal component of PPF seems to be defective, has suggested by data derived from studies in *C-met* (-/-) mice, demonstrating that diaphragmatic defects can be produced independently of myogenic process [Babiuk *et al.*, 2002]. The C-met protein, a receptor tyrosine kinase that is present on myogenic precursors, binds its ligand hepatocyte growth factor/scatter factor, signaling migration of these cells. Although the diaphragmatic musculature fails to form in the null mutants, the underlying connective tissue that comprises the amuscular substratum forms fully. These data provide a perspective on the mechanisms underlying CDH pathogenesis entirely novel from past theories. The focus now shifts from the muscularization of the diaphragm and closure of the pleuroperitoneal canals to understanding the mesenchymal amuscular component of the diaphragm.

In fact, CDH is commonly considered as a developmental abnormality but its exact molecular mechanism is unknown. Correct muscularization of the diaphragm requires complex coordination of precursor cells to delaminate, migrate, target diaphragmatic tissue, and differentiate; mutations in several genes involved in those processes have already been shown to cause aberrant muscularization. Until now, studies in KO mices stresses the role of several genes in diaphragm muscularization such as *C-met* [Babiuk, 2002], *Fog2* [Ackerman *et al.,* 2005], *Gab1* [Sachs *et al.,* 2000], *Gata4* [Jay *et al.,* 2007], *Lox* [Hornstra *et al.,* 2003], *MyoD* [Kablar *et al.,* 1998], Myogenin [Tseng *et al.,* 2000], *Pax3* [Li *et al.,* 1999], *RARa/RARb2* [Mendelsohn *et al.,* 1994], *Slit3* [Liu *et al.,* 2003] and *wt1* [Clugston *et al.,* 2006].

Interestingly, *Fog2* is the first gene recognized to be necessary for both primary lung development and primary diaphragm development providing evidence for the hypothesis that diaphragmatic defects may be associated with primary lung defects [Ackerman *et al.,* 2005]. This highlights the hypothesis that an insult occurs in a critical point of the embryonic development, affecting other organs than diaphragm, like the lung and even the heart.

3.2 Lung development

The development of the human lung encompasses a period starting with the appearance of the tracheal outgrowth from the foregut and ending in early childhood.

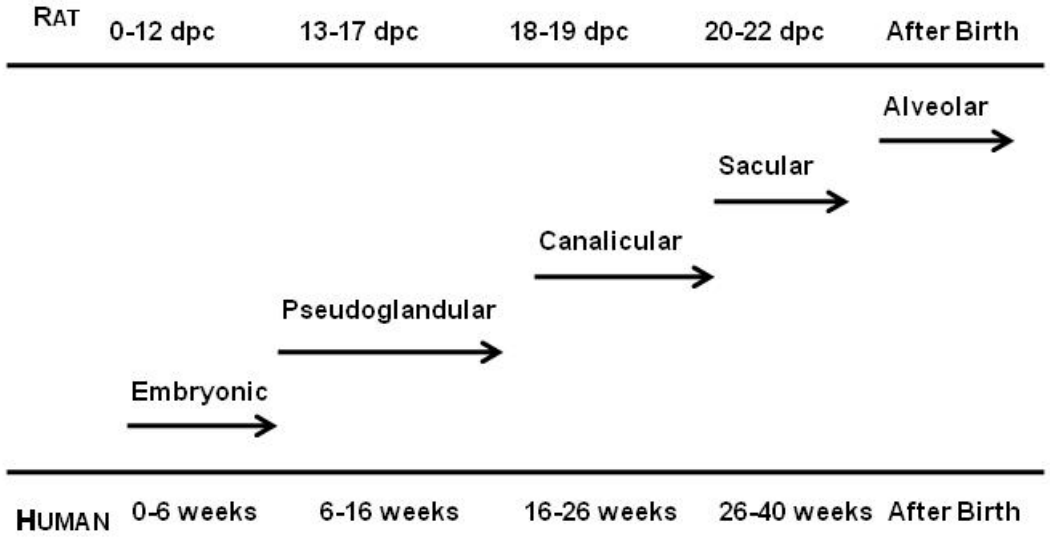


Figure 3. Lung development stages in rat and Humans. dpc, days post conception

The organogenesis of the lung can be divided into five distinct stages (Figure 3) [Post & Copland, 2002]. The early phases of human lung development comprises the embryonic (day 26 to 52 in human and 9.5 to 12 dpc in rat) and pseudoglandular (day 52 to end of 16 weeks of gestation and 13 to 17 dpc in rat) phases of lung

development, after which a hierarchical pattern is apparent, the prospective conductive airways have been formed, and the acinar limits can be recognized.

During the pseudoglandular phase the primitive airway epithelium starts to differentiate and neuroendocrine, ciliated and goblet cells appear. At this stage, mesenchymal cells have begun to form cartilage and smooth muscle cells. In humans, at 8 weeks, fetal breathing movements can be identified. In the subsequent canalicular phase (17 to 26 weeks in human and 18 to 19 dpc in rat), the airways branching pattern is completed and the gas-exchange region develop. During this period, respiratory bronchiole appears, interstitial tissue decreases, vascularization of peripheral mesenchyme increases, differentiation of the cuboidal epithelium into type I and type II cells occurs and begin the surfactant production. In the saccular (terminal sac) phase (24 to 36 week's gestation in human and 20 to 22 dpc in rat), the growth of the pulmonary parenchyma, the thinning of the connective tissue between airspaces, and the further maturation of the surfactant system are the most important steps towards *ex-utero* life.

At birth, although already functional, the lung is structurally immature, because alveoli, the gas-exchange units of the adult lung, are practically missing. The airspaces present are smooth-walled transitory ducts and the saccules with primitive septa are thick and contain a double capillary network. During the alveolar stage (36 week's gestation to term and at least 36 months postnatal in human and 20 to 22 dpc in rat), alveoli are formed through a septation process that greatly increases the gas exchange surface area.

Lung development is strictly regulated through reciprocal interactions between the epithelial and mesodermal mesenchymal layers, leading to the formation of the lung bud and branching morphogenesis, a reiterative process in which a network of

airways tubes is generated by the successive and invariant branching of the epithelium. When the embryo is approximately 4 weeks old, the primordium of the respiratory system appears as an outgrowth from the ventral wall of the foregut. During its separation from the foregut, the respiratory primordium forms a midline structure, the trachea, and two lateral outpocketings, the lung buds that progressively growth and branching in caudal and lateral directions. These processes are dependent on interactions between the foregut endoderm and its surrounding mesoderm. An interesting recent discovery links lung budding in foregut to cardiac development mesoderm. In fact, with regard to the lung localization in the foregut, the heart provides critical signals. Serls *et al.* found, using organ culture experiments, that lung specification is induced by adjacent cardiac mesoderm and the signals from this organ can be replaced with exogenous fibroblast growth factor (*Fgf*) 1 and 2 [Serls *et al.,* 2005].

Additionally, both lung budding and bronchial branching are dependent of retinoic acid (RA) signaling, which is ubiquiarious and maximal in endoderm, mesenchyme and primitive pleural tissues. Retinoic acid establishes a proximal type of cellular pattern, in which cells do not differentiate, keeping its potential to growth and branching. Further, the neighboring mesenchyme to the distal forming lung bud expresses *Fgf10,* essential for lung growth and branching. *Fgf10* induces bud formation and the bud elongates following the nidus of *Fgf10* as it migrates through the mesenchyme. Recent evidences suggest that *Fgf10* promotes both the proliferation of the endoderm and its outward movement. The mitogenic effect of *Fgf10* is antagonized by *Bone Morphogenic Protein* 4 (*Bmp4*) [Weaver *et al.*, 2000]. As bud outgrowth continues endodermal, *Bmp4* expressions increases. Meanwhile, *Fgf10* expression gradually decreases at the tip but is upregulated laterally and overlies proximal endoderm, leading to the outgrowth of two new buds. The interaction between mesenchyme and epithelium allow a fine regulation of the lung

development, specifically of the differentiation and the proximal-distal growth (Figure 4). Retinoic acid rule this local regulation, directly and indirectly thorough the *Sonic hedgehog* (Shh), inhibiting excessive expression of local fgf10 that could cause pulmonary immaturity [Malpel *et al.*, 2000]. Ectopic branching between the branches is probably also quenched by TGF beta and extracellular matrix. Excess branching and growth at the distal bud is downregulated by the induction of Sproutys to inhibit *Fgf* signaling [Warburton *et al.*, 2000; Cardoso, 2001; Cardoso & Lü, 2006].

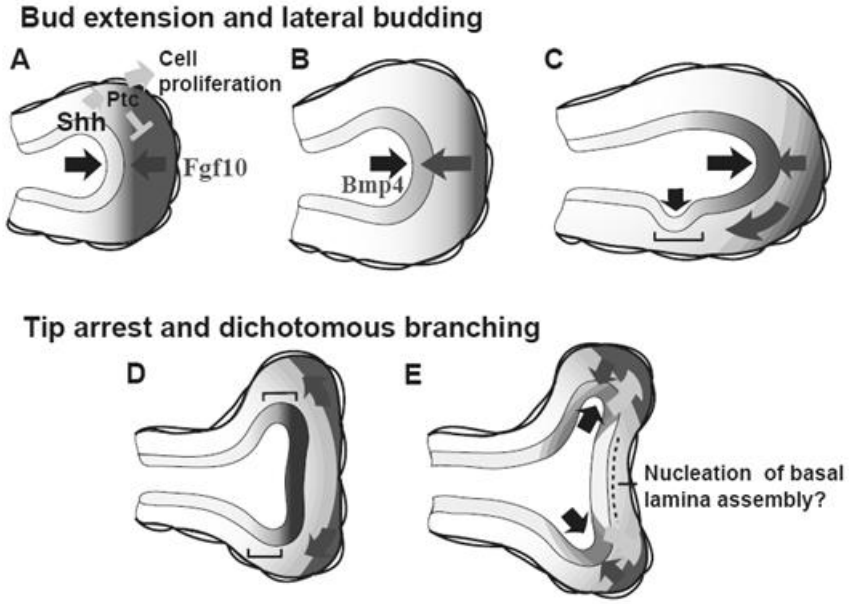


Figure 4. Model for the dynamic interaction of growth factors in lung bud morphogenesis. Throughout early development shh is expressed in the endoderm. (A) A bud shortly after initiation. Fgf10 is transcribed at high levels in distal mesenchyme but only very low levels of *Bmp4* expression are seen in the distal mesoderm. (B) As bud outgrowth continues, endodermal *Bmp4* expression increases. Meanwhile, *Ffg10* expression gradually decreases at the tip but is upregulated laterally. (C) As the Fgf10 expression domain moves laterally, it overlies proximal endoderm. (D) Before undergoing dichotomous branching, the distal endoderm expresses such high levels of Bmp4 that forward movement stops. (E) The mechanism that regulates Fgf10 at the tip now drives expression laterally and symmetrically, leading to the outgrowth of two new buds. The cycle of outgrowth, promotion of mesenchymal proliferation and endoderm movement begins again. (adapted from Weaver *et al.*, 2000)

Additionally, when lung branching, RA is also regulated and characterized by a proximal-distal gradient of RA activation, with less response in the distal

mesenchyme near sites of budding. This decreased response at distal sites can be ascribed to: i) diminished activity of RALDH-2 (retinaldehyde dehydrogenase), the major RA-synthesizing enzyme; ii) increased activity of COUP-FT2 (*chicken ovoalbumin upstream promoter-Transcription Factor*), responsible for inhibition of RA receptor; iii) increased RA degradation in the epithelium via P450RAI-mediated metabolism, an RA-inducible RA metabolizing enzyme of the cytochrome P450 family. During normal development, restriction of RA signaling in time and space is fundamental for correct expression of *Fgf10* and *Bmp4* and distal morphogenesis. Additionally, the RA gradient is critical for proximal-distal pattern formation [Wang *et al.*, 2006].

Pulmonary hypoplasia associated with CDH remains a major therapeutic problem. In CDH, a number of studies have investigated the underlying defect in these lungs. Gross congenital abnormalities of the lung are also seen [Nose *et al.*, 2000]. Using morphometric analysis, in 1951, Reid found that the airway number, and thus alveolar, acinar, and arterial number, were all reduced in both lungs. This defect was most obvious in the lung ipsilateral to the CDH [Reid, 1951]. A defect in the regulation of branching morphogenesis could occur at any stage to yield such a phenotype. Thus one could postulate defects in RA, *Shh*, and *Fgf* signaling among others. Indeed CDH has been identified in several models where these pathways have been altered, and the RA pathway has been deeply studied. Retinoid signaling has been implicated in CDH pathogenesis in animals and Humans. Congenital diaphragmatic hernia and lung hypoplasia has been described in vitamin A deficiency models in rats, retinoic acid receptor (RAR) alpha/beta compound knockouts, as well as in mice with the deletion of the retinoic acid receptor X (RXR) ligand activation domain [Wilson *et al.*, 1953; Mendelsohn *et al.*, 1994; Antipatis *et al.*, 1998; Mascrez *et al.*, 1998; Kling *et al.*, 2007]. In Human infants with CDH and lung hypoplasia, it was reported low plasma concentrations of retinol and retinol binding proteins and it

is account an association with chromosome 15q defects [Major *et al.*, 1998; Enns *et al.*, 1998]. A gene on chromosome 15 in the region of the deletion or translocation (15q24-26) encodes for cellular retinoic acid binding protein-1. Additionally, exogenous administration of vitamin A and RA decreases the incidence of CDH and improves lung growth in the experimental model of CDH [Thébaud *et al.*, 1999; Babiuk *et al.*, 2004; Montedonico *et al.*, 2006].

To explain the pathogenesis of pulmonary hypoplasia in CDH Keijer *et al*. postulated the dual-hit hypothesis, which explains pulmonary hypoplasia in CDH by two developmental insults. The first insult occurs early in gestation, before diaphragm closure, due to a still unidentified background of genetic and environmental factors. This insult affects both lungs during branching morphogenesis in a similar fashion. After defective development of the diaphragm, the second insult occurs due to the presence of abdominal organs in thoracic cavity and affects the ipsilateral lung only at a later stage of development [Keijer *et al*., 2000]. In this scenario, herniated abdominal organs will interfere with fetal breathing movements of the ipsilateral lung, resulting in a greater impairment of the development of the ipsilateral lung than the contralateral lung. An interesting property of hypoplastic lungs relates with its ability to grow, at least in vitro, at higher rates than normal lungs. In this phenomenon seems to be involved cytokines like interleukin 6 and neuroendocrine products like ghrelin [Nogueira-Silva *et al.*, 2006; Santos *et al.*, 2006].

In conclusion, defects in early and late stages of lung development are likely to lead to lung hypoplasia, an essential component of CDH. Indeed by understanding the factors that regulate lung and diaphragm formation and the interaction between both these structures during development, we will obtain new insights into this important congenital malformation.

3.4. Heart Development

The development of the vertebrate heart can be considered an additive process, in which additional layers of complexity have been added throughout the evolution of a simple structure (linear heart tube) in the form of modular elements (atria, ventricles, septa, and valves). In mammals, cardiac progenitors cells arise from endoderm associated splanchnic mesoderm and coalesce at the anterior of the embryo. These cells adopt a crescent shape with the apex of the crescent lying close to the anterior junction between embryonic and extra-embryonic tissue, and the lateral arms of the crescent extending caudally. Cells of the cardiac crescent move ventrally and fuse or coalesce into a linear heart tube. This tube is composed of endothelial cells shrouded by a myocardial epithelium. The heartbeat is initiated at around this time and blood courses through the heart tube in a caudal to cranial direction; therefore, the venous inflow of the heart is initially located caudally, and the arterial outflow tract cranially [Dunwoodie, 2007].

More recent tissue ablation, genetic ablation, and lineage labeling experiments in chick and mouse embryos have demonstrated the dynamic nature of heart tube formation, and the possible existence of two distinct heart fields [Buckingham *et al.*, 2005]. According to these models, cells of the first heart field form the cardiac crescent and begin to differentiate *in situ*. They undergo complex morphogenesis to form the primary heart tube. In mouse, the first heart field contributes largely to the future left ventricle (LV), forms parts of the atrioventricular canals as well as atria, and only has a small contribution to the future right ventricle (RV) [Zaffran *et al.*, 2004]. The second heart field is a population of undifferentiated myocardial progenitor cells that lies medial and caudal to cells of the crescent and, later, dorsal to the heart tube [Buckingham *et al.*, 2005]. These cells contribute to the outflow

tract, RV and parts of the LV and inflow region [Verzi *et al.*, 2005; Zaffran *et al.*, 2004].

The initiation of cardiac differentiation has been related with *tinman* gene in the fruit fly *Drosophila melanogaster*, and in vertebrates with the cardiac gene *Nkx2-5*. These genes encode an NK-class homeodomain-containing transcription factor and have important roles in the differentiation and morphogenesis of the early developing heart. In mice, *Nkx2-5* is required for terminal differentiation of cardiac myocytes that includes, in large part, the establishment or maintenance of a ventricular gene expression program. Its role in early cardiac differentiation is crucial for the normal growth of the embryonic myocardium, which is apparent in the poorly developed myocardium of mice lacking *Nkx2-5* and in the inability of this primitive cardiac structure to grow beyond the earliest stages of heart looping [Tanaka *et al.,* 1998]. The gene expression of *Nkx2-5* seems to be regulated by myocardin, a cardiac-specific transcription factor, a cardiac gene promoter via serum response factor-binding sites [Wang *et al.*, 2001].

The progressive differentiation of precardiac cells appears to be regulated by the GATA family of zinc finger–containing transcription factors, and three GATA family genes have been identified as being expressed in the developing heart: *gata4*, *gata5*, and *gata6* [Charron *et al.*, 1999]. These genes are also important in the movements of the paired progenitor pools that coalesce to form the linear heart tube [Koutsourakis *et al.*, 1999]. In earlier step in the migration of cardiac precursors the basic helix-loop-helix (bHLH) transcription factor MesP1 has been shown to be required [Saga *et al.*, 1999].

The heart tube grows by division of myocardial cells and by addition of cells to both poles of the heart [Buckingham *et al.*, 2005]. During the looping stages of cardiac

morphogenesis, the outflow region swings to the right as the heart adopts a spiral form. With further development, the inflow portion of the heart, which includes the common atrium, moves in an anterior and dorsal direction such that the inflow and outflow complexes converge. The formation of the specialized chamber myocardium becomes evident during looping as it balloons out from the outer curvature of the heart tube in discrete zones [Christoffels *et al.*, 2000].

Considerable remodeling of the primitive heart occurs, beginning from the end of looping stages. The interventricular septum, which will divide the ventricles, forms in a highly defined region encompassing the junction of future LV and RV through polarized growth of myocardial cells [Franco *et al.*, 2006]. The outer layers of the ventricles become highly proliferative, resulting in a thickening of the chamber walls to form the “compact layer”, important for building cardiac chamber mass and volume. Associated with non-chamber regions of the myocardium, endocardial cushions form, which initially act to direct independent patterns of blood flow for the pulmonary and systemic circuits [Moorman *et al.*, 2006]. In the atrioventricular canal, endocardial cushions are the precursors of the tricuspid and mitral valves, while in the outflow tract they form a scaffold for the aorticopulmonary septum which divides the outflow tract into the aorta and pulmonary artery, and forms the aortic and pulmonary valves.

Various promoter elements that restrict the expression of genes to the atrial or ventricular chambers have been identified. *Iroquois homeobox* gene 4 (*Irx4*) is a member of the Iroquois family of homeodomain-containing transcription factor genes. Expression of *Irx4* is restricted at all stages of development to the ventricular myocardium in all species examined [Christoffels *et al.*, 2000]. This gene is involved in the positive and negative regulation of chamber-specific *myosin heavy chain* gene expression in the ventricular myocardium. *Irx4* represses the atrium-specific *slow*

myosin heavy chain 3 promoter acting via interaction with a retinoic acid receptor /vitamin D receptor complex of proteins [Wang *et al.*, 2001]. *Irx4*-deficient mice have impaired cardiac function and develop cardiomyopathy. Another gene identified has regulator of ventricle-specific morphogenesis is the *Hey2* gene, a ventricle-specific transcription factor related to the Hairy family of bHLH transcription factors [Leimeister *et al.*, 1999]. Mice lacking Hey2 develop a severe postnatal cardiomyopathy.

The expression patterns and roles of the bHLH transcription factors dHand and eHand as well as the T-box transcription factor *Tbx5* illustrate the correlation between chamber-restricted roles for factors expressed in a specific domain of the developing heart. *dHand* and *eHand* exhibit complementary patterns of expression in the developing mouse heart, with *dHand* more strongly expressed in the RV than in the LV and eHand predominantly restricted to the LV [Thomas *et al.*, 1998]. Regarding *Tbx5* it is expressed initially throughout the cardiac mesoderm in its earliest stages, but its expression pattern is rapidly refined, first as a posterior-anterior gradient in the linear heart tube, until mid gestation, when it is restricted to the atria and LV [Bruneau *et al.*, 1999]. Regarding, cardiac septation the multi-type zinc finger transcription factor FOG-2 has been implicated as well retinoic acid receptors [Fosset *et al.*, 2001; Mendelsohn *et al.,* 1994].

Maturation of the heart into fully functional trabeculated chambers and septation of the atria and ventricles from one another and between their left and right sides are important processes that require precise integration of growth and differentiation signals. Defects in these processes account for the majority of congenital heart malformations in humans, including atrial and ventricular septal defects (ASD and VSD, respectively), tetralogy of Fallot (TOF), common atrioventricular canal, and double-outlet right ventricle. Genetic analysis of inherited cardiac septation defects

has shown that dosage-sensitive redeployment or sustained function of transcription factors required for early cardiogenesis (eg, *Nkx2-5* and *Tbx5*) is a major factor in septal morphogenesis. Dominant mutations in NKX2-5 have been found in patients with ASD, VSD, TOF, and Ebstein's anomaly of the tricuspid valve, often accompanied by conduction disease [Schott *et al.*, 1998]. The identification of TBX5 mutations in Holt-Oram syndrome has also provided insight into cardiac septation [Basson *et al.*, 1999].

Neural crest is essential for cardiac development, due to its rule in development of: i) branchial arches e derived structures, including arch aortic arteries that will originate the great vessels in the thorax; ii) ventricular outflow tract and septum. Cardiac neural crest cells require a wide variety of environmental signals in order to be specified in the neural tube and then to migrate, proliferate, differentiate and survive. Wnt, FGF, BMP and RA, are required for neural crest induction. Many transcription factors and signaling molecules have been implicated in the later steps of migration, proliferation, survival and differentiation of the cardiac neural crest. Cardiac neural crest ablation leads to a number of cardiovascular and non-cardiovascular defects. Non-cardiovascular phenotypes include hypoplasia or aplasia of the thymus, parathyroids and occasionally the thyroid gland. The cardiovascular phenotypes include three distinct components: i) defective development of the cardiac outflow tract including persistent truncus arteriosus and outflow misalignment, ii) abnormal patterning of the great arteries, and iii) abnormal myocardial function [Hutson & Kirby, 2007].

The association between CDH and congenital malformations is well defined by several authors, both in animal models [Migliaza *et al.* 1999] and in humans a wide spectrum of cardiovascular malformations associated with CDH been reported in the literature. These cardiac malformations are mainly truncoconal defects, as VSD,

TOF, truncus arteriosus, vascular rings and anomalies of ventricular outflow tract. In the animal model of CDH induced by the teratogen nitrofen, it is considered that nitrofen interferes with the normal neural crest cell migration, causing conutruncal heart malformations [Yu *et al.,* 2002]. Interestingly, previous studies demonstrated *in vitro* that nitrofen inhibits RALDH-2, the major retinoic acid-synthesizing enzyme that has recently been shown to play an important role in lung and *neural crest dependent* heart development [Mey *et al.*, 2003].

The prognostic value of heart morphology in CDH has been thorough studied. Several authors demonstrated that the presence of heart malformations significantly worsen the prognosis of the disease. Even in the absence of cardiac morphologic defects several authors suggests that the heart could be hypoplastic, as occurs in the lung, and this was suggested as a parameter of poor outcome in affected infants (Table 1). This heart underdevelopment should be secondary to LV hypoplasia [Siebert *et al*. 1984; Crawford *et al.*, 1989; Schwartz *et al.*, 1994; Thébaud *et al.*, 1997] and correlated with lung hypoplasia [Karamanoukian *et al.*, 1995]. However, the determinants of heart hypoplasia and its real significance were largely unknown and not consensual. In previous studies, we demonstrated in the experimental rat model of CDH the absence of heart hypoplasia at term of gestation, specifically LV hypoplasia [Correia-Pinto *et al.* 2000, 2003].

A possible explanation for these apparent contradictory data was recently purposed by Lin and colleagues. They consider that a slender compressed LV cavity is a common feature of congenital and acquired heart defects associated with pulmonary hypertensive vascular disease, deficient LV preload, or preferential RV volume loading [Lin *et al.*, 2007]. In these conditions, the enlarged RV compresses the LV which may be interpreted as LV ‘‘hypoplasia’’. Examples of other conditions with apparent LV ‘‘hypoplasia’’ but normal aortic and mitral valves and normal ventricular

Table 1. Prognostic of heart malformations and heart related parameters in CDH.

Authors	Main Conclusion	Subject	Timing
Kalache *et al*. 2007	Cardiac axis before fetal viability has no role in predicting postnatal outcome.	Human	Early gestation
Sokol et al. 2006	Antenatal branch PA size correlates with postmortem lung weight.	Human	Fetal
Graziano *et al.* 2005	Congenital heart disease (CHD) is with 10.6% of CDH. These patients have poor survival compared to non CHD.	Human	Newborn
Dillon *et al.* 2004	PH is a critical determinant of survival in CDH. Pulmonary artery pressure estimation predict clinical outcome.	Human	Newborn
Verklan *et al.* 2004	Heart rate variability as an indicator of outcome in CDH.	Human	Newborn
Correia-Pinto *et al.* 2003	Heart growth is impaired only early in gestation, due to nonmechanical factors. Heart weight predicts lung weight only in early gestational ages.	Rat	Throughout gestation
Cohen *et al.* 2002	Risk of death is higher in patients with CDH plus CHD than without CHD.	Human	Newborn
Correia-Pinto *et al.* 2000	CDH was not associated with heart underdevelopment.	Rat	Term
Tanabe *et al.* 2000	Doppler flow patterns through ductus arteriosus may be useful for predicting prognoses and selecting suitable treatment for CDH infants.	Human	Newborn
Suda *et al.* 2000	A modified McGoon is the most significant prognostic factor in CDH.	Human	Newborn
Miggliazza *et al.* 1999	Heart hypoplasia occurs in the rat model of CDH, but it is related with nitrofen.	Rat	Term
Baumgart *et al.* 1998	CDH nonsurvivors have lower LV mass, low LV output and cardiac malposition persisted despite CDH repair.	Human	Newborn
Thébaud *et al.* 1997	Fetal LV hypoplasia (ventricular disproportion) may be a predictor of outcome and of PH.	Human	Fetal
Karamanoukian *et al.* 1996	LV hypoplasia exist in CDH and correlates with lung weight.	Lamb	Fetal
Schwartz *et al.* 1994	LV mass index in CDH is significantly lower than in other causes of PH. LV mass may predict need for ECMO.	Human	Newborn
Sharland *et al.* 1992	Underdevelopment of left-sided heart and cardiac malformations are prognostic factors in CDH.	Human	Fetal
Momma *et al.* 1992	The heart is hypoplastic in fetal CDH.	Rat	Fetal
Crawford *et al.* 1989	Fetal ventricular disproportion is associated with poor survival.	Human	Fetal
Siebert *et al.* 1984	LV hypoplasia in CDH.	Human	Newborn

volumes include total anomalous pulmonary venous drainage, atrioventricular canal defect, arteriovenous malformations, and almost any cause of severe PH. In CDH, it seems likely that apparent LV ‘‘hypoplasia’’, especially in the presence of a normal aortic and mitral valve, is due to compression of an under-filled pre-load deficient LV by the hypertensive RV rather than true LV hypoplasia. Indeed, LV mass, LV size, aortic size, LV to RV ratio, and aortic to pulmonary artery discrepancy do not predict postnatal outcome in patients with CDH [Suda *et al.*, 2000; Sokol *et al.*, 2006].

In patients with CDH, it seems likely that severe lung hypoplasia and elevated pulmonary vascular resistance, which cause marked RV enlargement and the apparent left heart ‘‘hypoplasia’’ are the real modifiers of outcome. Indeed, indicators of lung hypoplasia such as branch pulmonary artery size, lack of branch pulmonary artery growth in utero, and postnatal lung area to head circumference ratio may be better predictors of mortality and morbidity than left ventricular volume [Cohen *et al.*, 2002; Sokol *et al.*, 2006]. Thus, the morphology of the left ventricle in CDH is more likely to be related to the state of the pulmonary vascular bed.

3.5 Development of pulmonary vascular system

The blood vessels in the lung are thought to arise from a combination of angiogenesis and vasculogenesis. Angiogenesis is believed to contribute to the formation of the proximal or central blood vessels in the lung and occurs through several important steps: breakdown extracellular matrix, sprouting of cells from preexisting vasculature, survival and proliferation of these cells, migration of cells away from existing vessels, morphogenesis to form tubes, and recruitment of accessory cells. The term vasculogenesis is used to describe *de novo* creation of blood vessels, in this case out of the primordial pulmonary mesenchyme. Endothelial cells in the mesenchyme are thought to arise from primitive angioblasts. The end

result of vasculogenesis is a primitive plexus of endothelial cells that surrounds the branching epithelial tubes and then remodels to form mature arteries, capillaries and veins. In the classical description of pulmonary vascular development, these two processes merge at 10 to 11 weeks of gestation in humans in the form of communicating channels that provide continuity of blood flow [Stenmark *et al.*, 2003].

Some investigators challenge the theory of proximal angiogenesis and distal vasculogenesis, claiming that the proximal vasculature is also derived via vasculogenesis. Support for these claims include studies that demonstrate continuity between central and peripheral lung vessels as early as 38 days of gestation in humans and others that show markers of endothelial cells in a continuous fashion from pulmonary arteries to the peripheral primitive vascular network, suggesting a common underlying process [Hal *et al.*, 2000].

Another area of relative uncertainty concerns the bronchial arteries. Although these vessels are known to accompany the airways and are thought to derive from direct angiogenic budding off the aorta, much less is known about the specifics of bronchial arterial development and remodeling. Presently, the precise mechanisms of these processes remain to be elucidated.

Critical molecular pathways determine the mechanisms of vascular development and could be altered in CDH disease. These determinants include transcription factors, peptide growth factors and receptors, cell adhesion receptors and intercellular adhesion molecules. Some of the most documented molecular mechanisms include vascular endothelial growth factor A (VEGF-A), endothelial monocyte activating polypeptide II (EMAP II), transforming growth factor-β (TGF-β) family, Wnt signaling pathway, forkhead box, insulin-like growth factors (IGF-I and -II), Notch signaling, ephrins, as well as angiopoietins and Tie receptors [Miniati, 2007].

As hypoxic environment is critical for vascularization, it is feasible that lung airway branching morphogenesis in utero is controlled by oxygen-regulated pulmonary vascular development. Recent reports have indeed suggested an active role for vascularization in lung development. In fact, epithelial branching morphogenesis *in vitro* was dramatically diminished when pulmonary vascular development was inhibited suggesting that vascular development might guide bronchial branching [van Tuyl *et al.,* 2005]. Interestingly, a low-oxygen environment enhances branching of both distal lung epithelium and vascular tissue and pulmonary vascular development appears to be rate limiting for epithelial branching morphogenesis [van Tuyl *et al.*; 2005].

Recently, our group detected that embryonic essential myosin light chain (MLC1a) and regulatory myosin light chain (MLC2) were absent in rat hypoplastic nitrofen-induced fetal lungs during pseudoglandular stage of lung development. We also found that MLC1a was expressed only in vascular smooth muscle cells of pulmonary artery, whereas MLC2 was present in peri-bronchic smooth muscle cells and vascular smooth muscle cells of pulmonary vessels. MLC expression is most likely regulated by retinoic acid metabolism, whereas disruption of MLC1a expression during early pulmonary development led to growth and branching impairment, entailing an important role in normal lung branching morphogenesis [Santos *et al.*, 2007]. These observations emphasize the idea that smooth muscle cells differentiation delay might translate into a deficiency in contractile protein content, which might have a role among the early molecular determinants of lung hypoplasia in CDH [Santos *et al.*, 2007].

Further in utero development occurs according to the surrounding environmental stimuli and in preparation for postnatal respiration and gas exchange. The fetal

pulmonary vasculature is a low-flow, high-resistance system, with corresponding medial and adventitial hypertrophy of the blood vessels. During the first two months of life in normal human newborns, this vascular wall thickening gradually remodels in response to the change to a high-flow, low resistance system. Other changes that occur before birth include a rapid increase in the number of pulmonary blood vessels during the third trimester and a phenomenon known as "intussusceptive angiogenesis," both of which account for the 30-fold expansion of the distal lung vasculature surface area that occurs after birth. Intussusceptive angiogenesis refers to growth of the capillary network "within itself" and has now been described to occur in many vascular beds in addition to the lungs [Haworth *et al.*, 2006].

Regarding pulmonary vessels tone, pulmonary expression of endothelial nitric oxide synthase (eNOS) increases throughout gestation, together with VEGF, and both factors are potent stimulants of pulmonary angiogenesis and vasorelaxation. In fetal lung, the concomitance of the increase in eNOS expression and the onset of alveolarization points to an important role of eNOS in airway maturation. The NO pathway is involved in angiogenesis, lung development, and vasorelaxation in human fetal lung. Despite this, during the perinatal period, when pulmonary vasodilation is maximal, endothelial NO synthase and VEGF are weakly expressed. This raises the possibility of the involvement of vasorelaxants other than NO at the time of birth. One candidate is endothelial-derived hyperpolarizing factor, which induces smooth muscle hyperpolarization by activating K_{ATP} channels. Another candidate is endothelin (ET)-1, together with its receptors ET-A and ET-B. ET-A receptors are located exclusively on smooth muscle cells and mediate vasoconstriction, whereas ET-B receptors mediate vasoconstriction when located on smooth muscle cells and vasodilatation when located on endothelial cells. In human fetal lung, K_{ATP} channels and ET-B receptors could be important in mediating the perinatal pulmonary vasodilation

crucial for adapting the pulmonary circulation to extrauterine life [Mohseni-Bod *et al.*, 2007].

At the time of birth, the pulmonary vasculature must dilate to accommodate the whole cardiac output. In CDH, this normal postnatal adaptation of the pulmonary vasculature does not happen. It seems that newborn pulmonary arteries continued to behave similar to the prenatal arterioles, i.e., they failed to respond to vasodilators, namely they exhibit a blocked NO-cGMP pathway [Vukcevic *et al.;* 2005]. In CDH occurs significant alteration in expression of ET-A receptors, VEGF and of K_{ATP} [Chang *et al.*, 2004; Sakai *et al.*, 2004; De Lagusie *et al.*, 2005]. There are also reports of higher prevalence of the active polymorphisms of the angiotensinogen converting enzyme and angiotensinogen genes, abnormal response of the arterioles to hypoxia and to oxygen, abnormal distribution of matrix matalloproteinases and tissue inhibitors of metalloproteinases, as well as increase in the ratio of the metabolites of thromboxane A2 to the metabolites of prostacycline [Nakayama *et al.*, 1992; Newell *et al.*, 1998; Solari *et al.*, 2004; Masumoto *et al.*, 2006].

An interesting aspect is related with the possible existence of fetal PH in CDH. Grover *et al.* demonstrated experimentally that fetal PH impairs vascular growth, which disrupts critical signaling pathways regulating lung vascular and alveolar development, thereby interfering with alveolarization and ultimately resulting in lung hypoplasia [Grover *et al.*, 2005]. However, no previous work investigated this hypothesis neither the impact of PH in fetal heart function in CDH.

4. ASSESSMENT OF PULMONARY HYPERTENSION

4.1. Pulmonary hypertension in newborns

The definition of pulmonary arterial hypertension in infants and children is the same as for adult patients. It is defined as a mean pulmonary artery pressure ≥25 mmHg at rest or ≥30 mmHg during exercise, with a normal pulmonary artery wedge pressure.

Whether PH is due to increased flow or resistance depends on its cause. By definition, hyperkinetic PH refers to pulmonary arterial hypertension from congenital systemic to pulmonary communications with increased pulmonary blood flow, e.g. VSD or patent ductus arteriosus. Pulmonary venous hypertension is caused by disorders of left heart filling, e.g. mitral stenosis, pulmonary venous obstruction or LV failure. Unless exist left heart obstruction or dysfunction causing pulmonary venous hypertension, in PH the pulmonary arterial wedge pressure is normal.

Persistent pulmonary hypertension of the newborn is a syndrome characterized by increased pulmonary vascular resistance, right–to-left shunting and severe hypoxemia. Persistent pulmonary hypertension of the newborn is frequently associated with pulmonary parenchymal abnormalities including meconium aspiration, pneumonia or sepsis, as well as occurring when there is pulmonary hypoplasia (as happen in CDH) or maladaptation of the pulmonary vascular bed in utero from unknown causes. In same instances there is no evidence of pulmonary parenchymal disease and the “injury” that is the trigger of the PH is unknown. Persistent pulmonary hypertension of the newborn is almost always transient, with infants either recovering completely without requiring chronic medical therapy or dying during the neonatal period despite maximal cardiopulmonary therapeutic

interventions. In contrast, patients with pulmonary arterial hypertension who respond to medical therapy appear to need treatment indefinitely.

The evaluation of PH severity is considered important for management of PH in general as well as for CDH infants. The assessment of PH is crucial to decide upon pulmonary vasodilator therapy as well as to monitor its effects. Furthermore, in CDH infants, the decision for surgical repair should be based on evidence of PH stabilization [Downard *et al.*, 2003]. However, since accurate evaluation of pulmonary artery pressure with Swan-Ganz catheter in newborns is not achievable, assessment of PH is mainly based on clinical and echocardiographic estimation, with its inherent limitations. Moreover, the establishment of more reliable parameters that could predict the PH severity and to screen early signs of PH-related heart dysfunction could particularly be useful to identify those newborns with more severe forms that could benefit from more aggressive treatment like ECMO.

4.2. Clinical and echocardiographic assessment of PH and cardiac function

Clinical assessment of PH in infants has some specificity because they have several intracardiac shunts. The right-to-left shunt at *foramen ovale* and *ductus arteriosus* indicate a severe PH. Thus, further hypoxia and PH manifests in infants by an increasing pre-to-post ductal gradient of Sat O_2 >10%. This method although simple and useful as screening method of PH, it has poor ability to stratify the severity of PH. Additionally, in clinical practice, is frequent the evaluation of the severity of PH by the oxygenation and ventilator indices, that have been proposed has prognostic parameters in CDH [Skarsgard *et al.*, 2005]. Nevertheless, these parameters could be dependent of others factors acting in gas exchange status.

In our days, most of the evaluation and monitorization of PH has being made with echocardiography, indirectly by assessment of RV repercussion of increased pulmonary vascular resistance. Noninvasive estimation of pulmonary artery systolic pressure (PASP) is important for the detection of PH in patients at risk, for monitoring the evolution of the disease, and to determine the effect of treatment. Several methods have been proposed to estimate pulmonary hemodynamic including PASP, diastolic pulmonary artery pressure (PAP), and mean PAP [Ullet *et al.*, 2007]. The principal approach involves the measurement of the peak velocity of tricuspid regurgitation (TR) and the Bernoulli equation to estimate PASP. However, TR signal cannot be recorded in all patients. Therefore, alternative and easy applicable methods to estimate PAP would be very valuable in those cases when TR spectral envelope is faint or when there is no TR. Doppler echocardiography also allows estimation of diastolic PAP by means of measuring pulmonary regurgitation velocity and mean PAP using RV outflow tract flow acceleration time.

The severity of PH could be indirectly evaluated by assessment of its repercussion in RV function, using echocardiography. Knowledge about the role of the RV in health and disease historically has lagged behind that of the LV. Less muscular, restricted in its role to pumping blood through a single organ, and less frequently involved in diseases of epidemic proportions such as myocardial ischemia, the RV has generally been considered a mere bystander, a victim of pathological processes affecting the cardiovascular system. Consequently, comparatively little attention has been devoted to how RV dysfunction may be best detected and measured, what specific molecular and cellular mechanisms contribute to maintenance or failure of normal RV function, how RV dysfunction evolves structurally and functionally, or what interventions might preserve RV function. Nevertheless, even the proportionately limited information related to RV function, its impairment in various disease states, like PH, and its impact on the outcome of those diseases, suggests that the RV is an important

contributor and that further understanding of these issues is of pivotal importance [Leite-Moreira, 1997; Correia-Pinto, 2003].

The RV exposed to pressure overload, due to PH, has an initial adaptive response of myocardial hypertrophy followed by progressive contractile dysfunction. Chamber dilatation ensues to allow compensatory preload and maintain stroke volume despite reduced fractional shortening. As contractile weakening progresses, clinical evidence of decompensate RV failure occurs, characterized by rising filling pressures, diastolic dysfunction, and diminishing cardiac output, which is compounded by TR due to annular dilatation and poor leaflet coaptation. The increased size and pressure overload of the RV also produce diastolic dysfunction of the LV. Thus, the function and size of the RV are not only indicators of the severity and chronicity of PH but impose an additional cause of symptoms and reduced longevity. Right ventricular function is the most important determinant of mortality in patients with PH. The specific mechanisms underlying the development of RV failure secondary to PH are unclear. For example, it is uncertain whether some patients develop RV myocardial ischemia, whether there is microvascular endothelial cell dysfunction, and whether or not myocytes undergo apoptosis. In severe, end-stage PH, the shape of the RV is changed from the normal conformation and RV wall stress and free wall thickness appear to be inversely related.

Implicit in the discussion of RV function and dysfunction is the notion that there are reliable means to its assessment. The measurement of RV function is difficult for many reasons, in part because of the interplay between intrinsic myocardial performance and RV loading conditions as well as due to the RV geometry. The development of load-independent markers of RV function is a worthwhile goal. In adults, several markers of RV dysfunction have been reported, with implications for clinical deterioration and mortality, in heart failure and PH, like RV ejection fraction,

RV dilatation, TR and Tei index [Tei *et al.*, 1997; Grignola *et al.*, 2006]. The extent to which any of these parameters are useful as outcome measures in clinical research or practice, namely in infants remains unclear.

Recently, tissue Doppler imaging (TDI) emerge has an additional instrument to evaluate heart function. It is an extension of conventional Doppler flow echocardiography and has been proven to be a useful and feasible clinic et al tool for assessing global and regional LV and RV systolic and diastolic function since its introduction in the early 1990s. Also, it recently has emerged as a new method useful for predicting right atrial and ventricular pressure as well as evaluation of RV systolic and diastolic function [Nikitin *et al.*, 2004]. Nevertheless, other objective observer-independent methods are warranted to diagnose and stratify PH in infants.

4.3. Biomarkers in Pulmonary Hypertension

Several recent studies coming out mainly from non-pediatric centers have demonstrated the clinical relevance of biochemical markers to evaluate and determine the prognosis in patients with heart failure and PH. Several molecules which can be measured in the blood or sometimes in other biological fluids are known to be elevated in PH. These biomarkers are closely related to the physiopathology of PH, and two components must be emphasized: i) *endothelium*, which directly suffers the aggression that triggers several anomalies that ultimately lead to persistent vasoconstriction; ii) *heart* muscle, mainly the RV that suffer the pressure overload. Both endothelium and myocardium release on plasma several proteins that reflect the severity of the disease. The endothelium release proteins that result from the aggression process, and the myocardium releases proteins in an attempt to balance the hemodynamics impact of PH. From several of these markers, components of natriuretic system, ET-1, troponin, uric acid and endogenous NO

synthase inhibitor asymmetrical dimethylarginine (ADMA) seem to be the most promising molecules.

B-type natriuretic peptide (BNP) is a hormone of predominantly ventricular origin produced and released in response to increased ventricular wall stress [Baugman *et al*., 2002]. NT-proBNP, the amino-terminal portion of the preprohormone, is secreted into the peripheral blood in equimolar portions to BNP, but it has a longer half-life and is easier to measure. In recent years, NT-proBNP has emerged as a very sensitive biochemical marker for ventricular dysfunction in adult heart failure, which plasmatic level could be used as a guide for the response to therapy and to predict prognosis [Bettencourt *et al.*, 2005]. However, in children the knowledge about the significance of plasma levels of NT-proBNP is still limited. In healthy children, studies have shown that NT-proBNP levels are elevated soon after birth reaching its peak at 24 hours of life decreasing thereafter up to four months and remaining unchanged until the age of 15 [Yoshibayashi *et al*., 1995; Fleming *et al*., 2001; Koch *et al*., 2003; Mir *et al*., 2003; Nir *et al*., 2004]. NT-proBNP levels are elevated in children with congenital heart disease or cardiomyopathy [Suda *et al,* 2003; Kunii *et al*., 2003; Westerlind *et al.,* 2004]. In infants, it was also demonstrated that NT-proBNP is elevated in symptomatic patent ductus arteriosus in preterms [Holmstrom *et al*., 2001, Choi *et al*., 2005] and PH [Reynolds *et al*., 2004].

Endothelin-1 is a potent vasoconstrictor peptide derived from endothelial cells that is also produced by cardiac myocytes [Suzuki *et al*., 1993]. ET-1 induces myocardial cell hypertrophy and has a potent positive (ET-A receptors) and negative (ET-B receptors) inotropic and chronotropic effects on isolated heart muscle. The production of ET-1 in the heart is increased in pressure overload as in PH [Shah, 2007].

Cardiac troponin T is a specific cardiac troponin T (cTnT) and a marker of cardiomyocyte injury which is detectable when either the LV or the RV is injured. Torbicki and colleagues have recently evaluated the prognostic value of this biochemical parameter in adult patients with PH and found that despite similar cardiac hemodynamic, patients with higher levels of cTnT had worse survival [Torbicki *et al.*, 2003]. It is therefore likely that cTnT is a marker of excessive stress to the RV, and the authors suggest that cTnT may be of use in making therapeutic decisions in adult's patients.

Uric acid may be elevated in the blood of patients with chronic hypoxic conditions such as heart failure or lung disease. The elevation is thought to be the consequence of impaired oxidative metabolism of the tissue, with increased degradation of adenine nucleotides such as ATP. Uric acid is known to be a risk factor for long term mortality in heart failure. Uric acid has been found to be elevated in the serum of patients with idiopathic PH, and correlates with pulmonary vascular resistance [Nagaya *et al.*, 1999].

Similarly, in adult settings, some researchers investigated the potential role of the ADMA in patients with idiopathic pulmonary arterial hypertension and concluded that increased ADMA plasma levels are associated with unfavorable pulmonary hemodynamic and worse outcome in patients with PH (Kielstein *et al.*, 2005].

AIMS

This dissertation was planned aiming to unravel certain aspects of perinatal heart development and adaptation to CDH. Thus, the specific aims of these studies are described bellow:

i. Evaluate the effect of fetal treatment with vitamin A in lung growth of CDH fetuses, in the nitrofen rat model (study n.º 1).

ii. Investigate the existence of cardiac immaturity in the experimental rat model of nitrofen induced CDH during perinatal development (study n.º 2).

iii. Establish the normal perinatal genetic expression pattern of molecular ventricular overload markers and adaptations in CDH, in the experimental nitrofen induced rat model of CDH (study n.º 3).

iv. In Human infants submitted to cardiac catheterization evaluate the correlation between NT-proBNP and RV pressure (study n.º 4).

v. In Human CDH newborns, clarify the heart function adaptation to pulmonary hypertension and to ascertain the usefulness of ventricular overload biomarkers as prognostic indices (study n.º 5).

vi. To contribute for the establishment of a novel perinatal cardiovascular assessment protocol to apply in infants with CDH.

CHAPTER II

EFFECT OF ANTENATAL TREATMENT WITH VITAMIN A ON PULMONARY GROWTH IN CONGENITAL DIAPHRAGMATIC HERNIA

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Antenatal vitamin A administration attenuates lung hypoplasia by interfering with early instead of late determinants of lung underdevelopment in congenital diaphragmatic hernia

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Index words:
Lung development;
Retinoic acid;
Nitrofen rat model;
CDH

Abstract

Background/Purpose: Early and late lung underdevelopment in congenital diaphragmatic hernia (CDH) is likely caused by nonmechanical (directly mediated by nitrofen) and mechanical (mediated by thoracic herniation) factors, respectively. The authors investigated if vitamin A enhances lung growth because of effects on both early and late determinants of lung hypoplasia.

Methods: Twenty-seven pregnant Wistar rats were exposed on embryonic day (E)9.5 to 100 mg of nitrofen or just olive oil. From nitrofen-exposed pregnant rats, 12 were treated at day 9.5 or 18.5 with 15,000 IU of vitamin A. Lungs were harvested at E18, E20, and E22, weighed, and analyzed for DNA and protein contents. Left and/or right lung hypoplasia was estimated by assessment of the ratios of lung to body weight and left to right lung weight. Fetuses were assigned to 5 experimental groups: baseline (exposed neither to nitrofen nor vitamin A), nitrofen (exposed to nitrofen without CDH), CDH (exposed to nitrofen with CDH), nitr+vitA (exposed to nitrofen without CDH and treated with vitamin A), and CDH+vitA (exposed to nitrofen with CDH and treated with vitamin A).

Results: Incidence of hernia was significantly reduced in fetuses treated with vitamin A. When vitamin A was administered at E9.5, the authors observed similar effect on lung hypoplasia measured through

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ratio of lung to body weight at E18 in the nitrofen and CDH groups (nitrofen 1.92% ± 0.05%, CDH 1.92% ± 0.04%), whereas lung hypoplasia was attenuated relative to baseline (2.45% ± 0.05%) in 5% and 4% in nitrofen (nitr+vitA 2.05% ± 0.03%) and CDH (CDH+vitA 2.08% ± 0.04%) groups, respectively. At E20, lung hypoplasia was increased in CDH compared with nitrofen groups (nitrofen 2.52% ± 0.1%, CDH 2.39% ± 0.05%), whereas vitamin A attenuated lung hypoplasia, in relation to baseline (3.20% ± 0.07%), 14% in both nitrofen-exposed groups (nitr+vitA 2.96% ± 0.03%, CDH+vitA 2.83% ± 0.03%). At E22, lung hypoplasia was significantly higher in CDH group than nitrofen group (nitrofen 2.13% ± 0.06%, CDH 1.48% ± 0.03%), whereas lung hypoplasia was attenuated in 9% of both nitrofen-exposed groups (nitr+vitA 2.35% ± 0.06%, CDH+vitA 1.69% ± 0.05%) in relation to baseline group (2.38% ± 0.04%). Administration of vitamin A at E18.5 produced no significant effects on lung growth.

Conclusions: The authors conclude from these results that antenatal administration of vitamin A attenuates lung hypoplasia in CDH by interfering with early determinants of lung underdevelopment. This finding may have clinical implications because prenatal diagnosis of human CDH commonly occurs after 16 weeks' gestation when late determinants of lung hypoplasia likely predominate.

Despite improved understanding of the pathophysiology of congenital diaphragmatic hernia (CDH) and advances in perinatal care, morbidity and mortality remain significant [1-3]. The leading causes of mortality in CDH are pulmonary hypoplasia and severe persistent pulmonary hypertension [2].

Regardless of experimental and clinical efforts using new modalities directed toward reduction of pulmonary hypertension and/or improved pulmonary gas exchange in CDH infants [4-9], there has been relatively little impact on survival of the most severely effected subset of CDH infants [1,2]. Antenatal therapies that promote lung growth before birth remain an appealing approach for fetuses with severe CDH. Fetal surgical intervention is invasive, technically demanding, and limited by the maternal and fetal risks [10,11]. In fact, it was recently demonstrated, in a controlled randomized trial, that tracheal occlusion did not improve survival or morbidity rates in human fetuses with CDH, when compared with standard postnatal care [12]. Therefore, less invasive approaches such as antenatal pharmacological treatment to stimulate lung growth and maturation have been considered [13-17].

Retinoids and retinoic acid are vitamin A compounds that function as essential signals for growth and differentiation during lung and diaphragmatic development through binding to its receptors: retinoic acid receptor (RAR) and retinoid X receptor (RXR) [18-20]. Genetic analysis of the function of the various RARs and RXRs, in the mouse fetal lung, has been shown to have lung hypoplasia and/or diaphragmatic defect [21-23]. In human beings, the expression of RXR occurs in both proximal (epithelia and mesenchyme of the trachea and bronchi associated with cartilage) and distal (epithelia and mesenchyme of smaller distal bronchi) sites in fetal lung, whereas RAR is detected in distal mesenchymal lung cells [24]. In addition, several studies suggest that retinoids may have an important role in the pathogenesis of CDH [21,25-27]. Thébaud et al demonstrated in the nitrofen rat model that early administration of a single dose of prenatal vitamin A increases survival, decreases the incidence of CDH, and increases lung growth in nitrofen-induced CDH [14].

In the nitrofen-induced rat model of CDH, it has been previously demonstrated that lung hypoplasia, in contrast to heart hypoplasia [28,29], has distinct early and late gestational determinants. Early determinants appear to be directly mediated by nitrofen [28,30,31] because similar degrees of lung hypoplasia are observed at early gestational time points with or without concomitant CDH. Late determinants, likely because of mechanical factors caused by thoracic herniation, only become manifest after embryonic day (E)18 of gestation as we have previously demonstrated [28]. In human beings, prenatal diagnosis typically occurs relatively late in gestation (after 16 weeks) [32,33]. Analogy to the nitrofen-induced model would imply that lung hypoplasia occurring after prenatal diagnosis would be predominantly caused by late determinants.

The purpose of the present study was therefore to clarify if the ameliorating effects of antenatal vitamin A administration occurred during the early or late determinants of lung hypoplasia in CDH. This question would be of greatest relevance in consideration of timing and efficacy of antenatal treatment of human fetuses with vitamin A.

1. Materials and Methods

1.1. Animal model

Wistar female rats (225 g, Charles River, Barcelona) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. The rats were mated and checked daily for introital plugging. The day of plugging was defined as gestational day 0 for time dating. Twenty-seven pregnant rats were treated at day 9.5 of gestation either with a dose of 100 mg of nitrofen

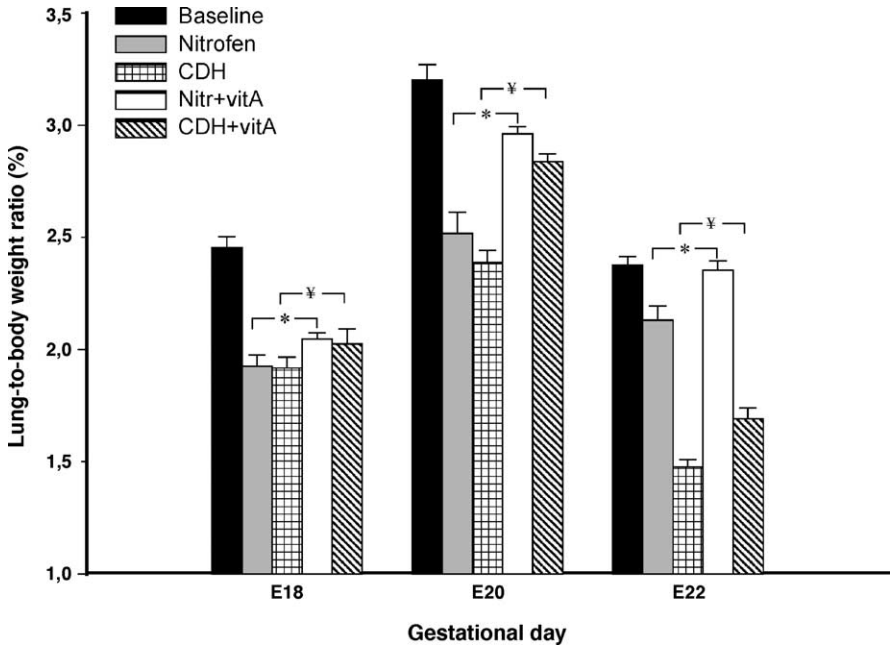

Fig. 1 Ratio of lung to body weight in baseline, nitrofen-exposed fetuses without CDH (nitrofen), nitrofen-exposed fetuses with CDH (CDH), and vitamin A–treated groups (nitr+vitA and CDH+vitA). In all nitrofen-exposed fetuses, a significant reduction in ratio of lung to body weight was observed compared with the baseline group at the same gestational time point. Early (E9.5) antenatal administration of vitamin A enhanced lung growth in all studied groups ($P < .05$: *nitr+vitA vs nitrofen; ¥CDH+vitA vs CDH).

dissolved in 1 mL of olive oil administered by gavage or with an equal volume of olive oil alone.

1.2. Vitamin A administration

The administration of vitamin A was performed in 12 time-dated pregnant rats treated with nitrofen. Two different protocols were performed: in the first protocol, vitamin A was administered at E9.5, whereas in the second protocol, vitamin A was administered at E18.5. In both protocols, vitamin A (15,000 IU; A-Vite, J Neves, Portugal) was diluted in 1 mL of olive oil and given through an oral gastric tube. The dose of vitamin A was chosen according to Wilson et al [27] as within the therapeutic range. Care was taken to avoid photo degradation of the vitamin A by ensuring that fresh vials of vitamin A were used and that the oral gastric tube was protected from light.

1.3. Experimental design

Pregnant rats treated with nitrofen alone or nitrofen and early (E9.5) vitamin A were killed at E18, E20, and E22 (term gestation E22). Pregnant rats treated with vitamin A late (E18.5) in gestation were killed only at E22.

From both protocols and at each time point, fetuses were harvested by cesarean section, and the body weights were measured on a precision balance (Scaltec Instruments, SBC 21, Heeligeesdadt, Germany). Under binocular microscopy (Leica, Wild M651.MS-D, Herbrugg, Switzerland) and after fetal decapitation, a laparotomy was performed to inspect the diaphragm. Through a median sternotomy, the lungs were excised, and the wet weights of each lung were then measured. Fetuses where assigned to 5 experimental groups: baseline (exposed neither to nitrofen nor vitamin A), nitrofen (exposed to nitrofen without CDH), CDH (exposed to nitrofen with CDH), nitr+vitA (exposed to nitrofen without CDH and treated with vitamin A), and CDH+vitA (exposed to nitrofen with CDH and treated with vitamin A).

1.4. Biochemical studies

Samples from left lung (LL) were weighed, snap frozen in liquid nitrogen, and stored at −80 °C until processing for determination of tissue protein and DNA. Protein content was determined by using an array spectrophotometer (model Jasco 7850 UV, Japan) with a modified micro-Lowry method assay (Sigma-Aldrich, Mo, USA). Bovine serum albumin (Sigma Chemical) was used as the standard. DNA was extracted from each sample according to the recommended protocol using the Quantum Prep Aqua Pure Genomic DNA kit (Biorad, Calif, USA).

1.5. Measurements

Wet weights of both lungs were measured, and the weights were expressed as a percentage of corresponding fetal body weight. Total DNA and protein contents of the LL were calculated and normalized for fetal body weight.

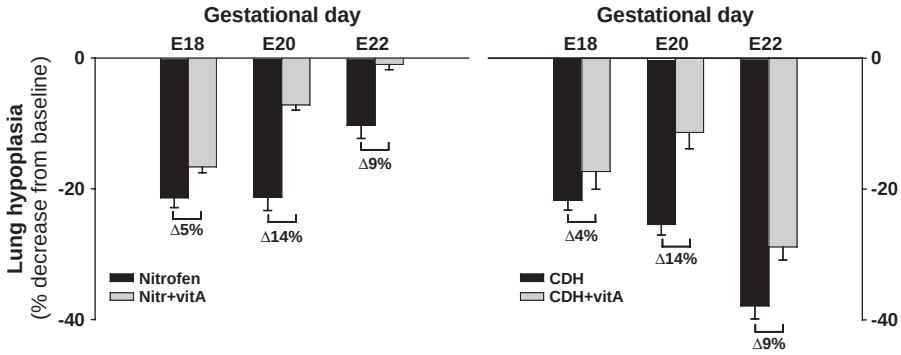

Fig. 2 Effects of vitamin A (E9.5) on lung hypoplasia throughout gestation. Nitrofen and CDH groups show divergence in lung hypoplasia as gestation proceeds. Whereas nitrofen-induced lung hypoplasia decreases as gestational age progresses, CDH-induced LL hypoplasia increases. At each time point of gestation, prenatal administration of vitamin A ameliorated the lung hypoplasia to a similar extent in the nitrofen and CDH groups.

For each gestational time point and experimental group, lung hypoplasia was estimated using the following formula: $[(\text{lung-to-body weight ratio})_{\text{group}} - (\text{mean lung-to-body weight ratio})_{\text{baseline}}]/(\text{mean lung-to-body weight ratio})_{\text{baseline}} \times 100\%$. The ratio of left to right lung weight was calculated for each fetus using the formula: (LL weight)/(right lung weight).

1.6. Statistical analysis

All quantitative data are presented as mean values ± SE. The different data sets of baseline, nitrofen, CDH, nitr+vitA, and CDH+vitA groups failed in the Kolmogorov-Smirnov test for normality. Therefore, statistical analysis was performed by the 2-way analysis of variance on ranks, and the Dunn test for posttest analysis. Statistical significance was set at $P < .05$.

2. Results

A total of 439 rat fetuses, distributed through the 3 gestational time points, were analyzed: 114 in the baseline group, 153 exposed only to nitrofen, and 172 exposed to nitrofen and vitamin A at E9.5. In addition, 24 nitrofen-exposed rat fetuses treated with vitamin A at E18.5 were analyzed at E22 and compared with nitrofen-exposed fetuses at the same gestational time points.

2.1. Early (E9.5) administration of vitamin A

The incidence of hernia was significantly reduced in fetuses treated with vitamin A (incidence of CDH: 50% in nitrofen-exposed fetuses, 22% in nitrofen-exposed fetuses treated with vitamin A).

Results from lung development are graphically represented in Fig. 1. At E18, the ratio of lung to body weight was similar in both nitrofen-exposed groups, but significantly lower than in the baseline group. Antenatal treatment with vitamin A improved the ratio of lung to body weight in both nitrofen-exposed fetuses. However, in comparison to baseline group, treatment with vitamin A completely ameliorated the reduction in ratio of lung to body weight by similar percentage: 5% and 4% in the nitrofen and CDH groups, respectively (Fig. 2).

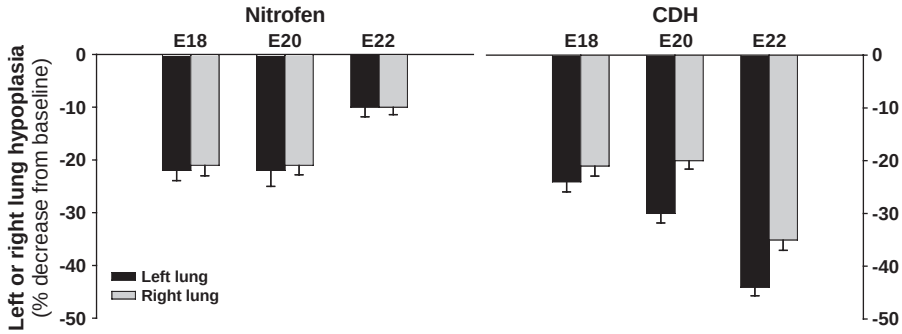

Fig. 3 Left and right lung hypoplasia in nitrofen and CDH groups. In the nitrofen group, lung hypoplasia is similar throughout gestation in right and left lungs, even if the degree of such hypoplasia decreases at E22. In the CDH groups, lung hypoplasia is similar for right and left lungs at E18 and nonsignificantly different from the nitrofen group at this gestational age. From E20 on, CDH fetuses show a bigger hypoplasia on left than on right lung, which is also significantly bigger than in nitrofen group.

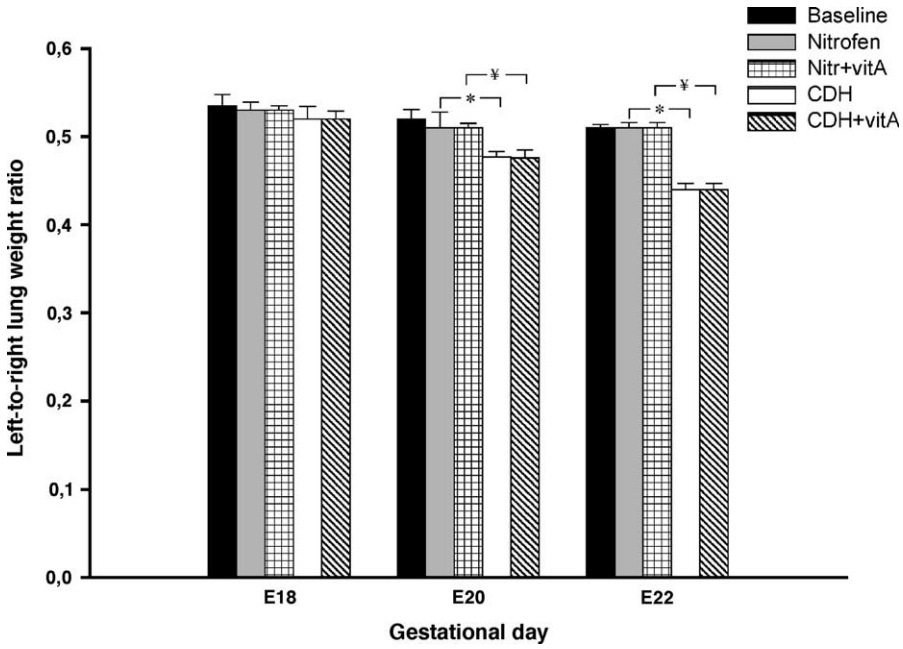

Fig. 4 Ratio of left to right lung weight before and after treatment with vitamin A (E9.5) ($P < .05$: *nitrofen vs CDH; ¥nitr+vitA vs CDH+vitA).

At E20, there began to be more lung hypoplasia in the CDH than in the nitrofen group. The ratio of lung to body weight was 21% and 25% lower than in the baseline group in the nitrofen and CDH groups, respectively. At this time point, prenatal treatment with vitamin A had a tremendous effect on the ratios of lung to body weight in fetuses from both nitrofen-exposed groups. In fact, treatment with vitamin A improved ratio of lung to body weight by a similar proportion (14%) in both nitrofen-exposed groups (Fig. 2).

Table 1 Developmental changes in LL protein, DNA, and protein/DNA ratio of baseline rats, nitrofen-exposed rats, and vitamin A–treated rats

	Gestational day		
	E18	E20	E22
LL protein/BW (mg/g)			
Baseline	0.84 ± 0.03	0.89 ± 0.04	0.68 ± 0.02
Nitrofen	0.49 ± 0.04[a]	0.71 ± 0.04[a]	0.54 ± 0.02[a]
CDH	0.48 ± 0.02[a]	0.60 ± 0.04[a]	0.43 ± 0.02[a]
Nitr+vitA	0.62 ± 0.02	0.91 ± 0.04[b]	0.65 ± 0.05[b]
CDH+vitA	0.52 ± 0.02	0.80 ± 0.03[c]	0.53 ± 0.04[c]
LL DNA/BW (μg/g)			
Baseline	90 ± 4	104 ± 6	59 ± 3
Nitrofen	55 ± 7[a]	79 ± 5[a]	46 ± 2[a]
CDH	52 ± 2[a]	76 ± 3[a]	38 ± 3[a]
Nitr+vitA	64 ± 3	105 ± 3[b]	56 ± 5[b]
CDH+vitA	53 ± 3	86 ± 1[c]	44 ± 1[c]
Protein/DNA ratio			
Baseline	9.6 ± 0.5	8.5 ± 0.3	11.8 ± 0.6
Nitrofen	9.7 ± 0.1	9.5 ± 0.6	12.0 ± 0.5
CDH	9.4 ± 0.5	8.9 ± 0.7	12.2 ± 0.1
Nitr+vitA	9.7 ± 0.4	8.9 ± 0.4	11.8 ± 0.9
CDH+vitA	9.8 ± 0.9	9.3 ± 0.8	12.2 ± 0.9

BW indicates body weight.
[a] $P < .05$: versus baseline.
[b] $P < .05$: versus nitrofen.
[c] $P < .05$: versus CDH.

By the end of gestation (E22), lung hypoplasia was significantly worse in the CDH group (38% lower than the baseline group) than in the nitrofen group (10% lower than the baseline group). Interestingly, vitamin A treatment resulted in improvement of the ratio of lung to body weight, which represented an attenuation of lung hypoplasia of 9% in both groups (Fig. 2).

Regarding the behavior of left and right lung development, we evaluated left or right lung hypoplasia by correspondent ratios of lung to body weight (Fig. 3) as well as ratio of left to right lung weight (Fig. 4). We observed that both lungs suffered from a similar degree of hypoplasia at E18 in both the nitrofen and CDH groups (Fig. 3). Consequently, no significant differences were observed in ratio of left to right lung weight among all studied groups at E18 (Fig. 4). At E20, the degree of LL hypoplasia, in the CDH group, increased to 32%, whereas the right lung hypoplasia remained similar to that observed at E18 (approximately 20%) (Fig. 3). Therefore, in contrast to E18 where the ratio of left to right lung weight was similar, at E20, there was a significant decrease of ratio of left to right lung weight primarily because of LL underdevelopment (Fig. 4). At E22, lung hypoplasia in the CDH group increased for both lungs, although it remained more pronounced in the left side (Fig. 3). Thus, ratio of left to right lung weight was significantly reduced in the CDH group. Interestingly, antenatal administration of vitamin A

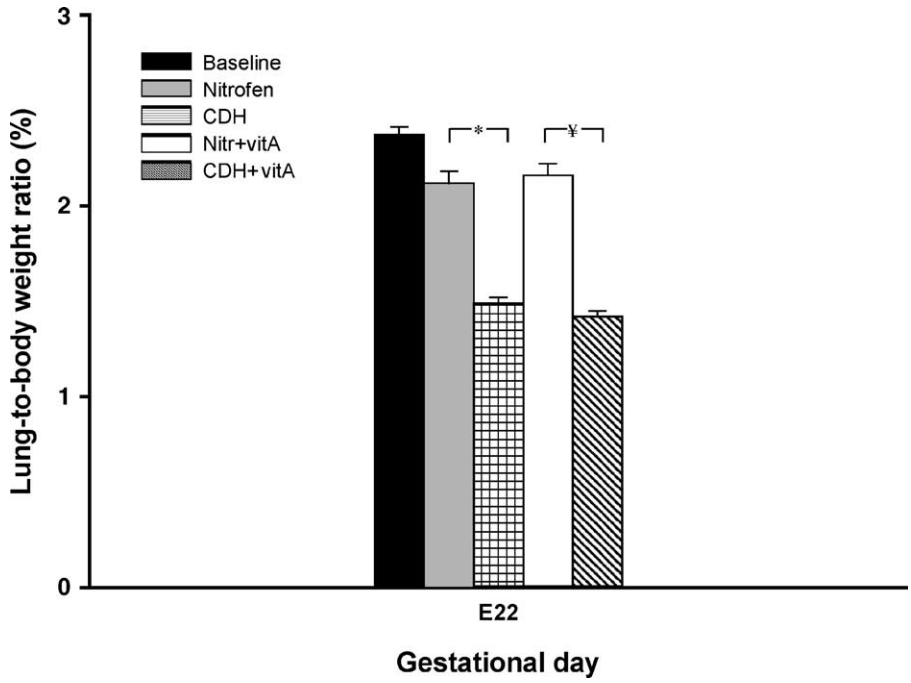

Fig. 5 Ratio of lung to body weight in before and after late-gestational (E18) treatment with vitamin A. Late antenatal administration of vitamin A did not affect lung growth ($P < .05$: *nitrofen vs CDH; ¥nitr+vitA vs CDH+vitA).

did not change these ratios at any gestational time point (Fig. 4). These results demonstrated that the effect of vitamin A on lung growth is of similar magnitude in both lungs.

Biochemical analysis of lung protein and DNA content (normalized to body weight) confirms the results obtained from morphological studies. Meanwhile, there was no significant difference in the DNA/protein ratio between groups that were treated or not treated with vitamin A (Table 1).

2.2. Late (E18.5) administration of vitamin A

Late administration of vitamin A did not affect the incidence of CDH in fetuses exposed to nitrofen. Moreover, analysis of ratio of total lung to body weight at E22 revealed no effect of late vitamin A administration (Fig. 5).

3. Discussion

In the rat nitrofen-induced CDH model, it has been previously demonstrated that lung hypoplasia has distinct early and late gestational determinants [30,31]. Early determinants appear to be directly related to the chemical effects of nitrofen, whereas late determinants seem to be primarily related to mechanical factors mediated by visceral thoracic herniation. This 2-hit hypothesis for lung hypoplasia, although not yet demonstrated to be relevant to human CDH, would have profound implications for antenatal pharmacological therapy. Because human CDH is rarely diagnosed before 16 weeks' gestation [32,33], early determinants of lung hypoplasia would essentially be completed by the time the diagnosis is made. Thus, it would be too late for pharmacological manipulations that counteract the nonmechanical phase of the pathophysiology.

Several previous studies have suggested that retinoids might have an important role in the pathogenesis of CDH [18,19,21,24-26]. As early as 1953, Wilson et al [27] demonstrated that the offspring of pregnant rats maintained on a vitamin A–deficient diet have a high incidence of CDH. Studies of retinoid receptors double null–mutants mice, lacking both α and β subunits of RAR, have demonstrated that a significant proportion of the offspring has lung agenesis/hypoplasia and/or diaphragmatic defect [21]. Furthermore, infants with CDH have 50% less plasma retinol and retinol-binding protein than age-matched controls [34]. More recently, it was proven that nitrofen-induced CDH is secondary to the inhibition of retinal dehydrogenase (RALD2), a key enzyme necessary for the production of retinoic acid, which is expressed in the developing lung and diaphragm [25]. All these evidences suggest that, in the CDH, there are an important role of retinoids signaling and that, in the CDH rat nitrofen-induced model, the disturbed molecular pathway is the same that is abnormal in human fetuses with CDH. Evidence for this is tightly supported from several references to an association of CDH with chromosome 15q defects, which encodes for cellular retinoic acid–binding protein-1 [26]. Thébaud et al [14,15] demonstrated, in the nitrofen rat model, that a single dose of prenatal vitamin A increases survival, decreases the incidence of CDH, and increases lung growth. All these previous studies were designed to evaluate the effect of early administration of vitamin A at term gestation [14,15]. Such an approach did not have adequate discrimination to

allow investigation of the effect of antenatal vitamin A on early and late determinants of lung hypoplasia.

We have previously demonstrated in the rat nitrofen-induced CDH model that late determinants of lung hypoplasia begin to influence lung growth after E18 [28]. These determinants are probably related to the mechanical effect on the lung, secondary to the presence of herniated viscera in the thorax. This hypothesis is supported by lung mechanotransduction studies [35-38]. In fact, growth and maturation of fetal lung are regulated by humoral and physical factors. Mechanical stretch, mediated by fluid-derived airways expansion and fetal breathing movements, effectively regulates the responsiveness of cells to growth factors by up-regulating the expression of related receptors, which can further mediate cell proliferation through autocrine and/or paracrine mechanisms [35-38]. We believe that late determinants of lung hypoplasia are related to interference with this mechanical (physical) forces acting on lung in late gestation. In contrast, determinants related to the molecular effect of nitrofen in the retinoid signaling pathway act early in gestation, interfering not only with lung development but also with heart and diaphragm development.

In the current study, we could assess the effect of administration of vitamin A on lung growth, during the 3 stages of lung development: pseudoglandular (E18), canalicular (E20), and alveolar (E22) [39]. We confirmed that lung hypoplasia was similar in both nitrofen-exposed at E18. Only at E20 could a difference in lung hypoplasia between nitrofen and CDH groups be demonstrated, and this difference only became clearly significant at E22 (Figs. 1 and 2). The appearance of this divergence in lung growth between the nitrofen and CDH groups marks the beginning of the mechanical determinants of lung growth in this model [28].

Interestingly, when we examined right and left lung development independently during gestation, it was clear that left and right lung development is differentially influenced by late determinants. The hypoplasia induced by mechanical effects becomes apparent at different gestational ages. Because we interpret a reduction in lung size in the CDH group compared with the nitrofen group to mark the beginning of the mechanical phase of reduction in lung growth in this model, the observation of a differential in LL hypoplasia at E18 versus a differential in right lung hypoplasia at E22 (Fig. 3) clearly suggests that mechanical effects influence LL development much sooner than right lung development. In this context, we propose that ratio of left to right lung weight can be used as a direct index to monitor the influence of mechanical factors. In fact, as analyses of Fig. 4 suggests, this index is only significantly influenced by the presence of CDH. Over the interval E18 to E22, we observed that the effect of CDH on this index increases progressively, which should be attributable to the increasing influence of mechanical factors on lung development in CDH fetuses as gestation proceeds.

Antenatal administration of vitamin A (E9.5) produced beneficial effects at all studied gestational time points. In fact, our study confirms the findings of Thébaud et al which demonstrate a clear attenuation of lung hypoplasia, expressed as ratio of lung to body weight and lung DNA and protein content after early treatment with vitamin A. This improvement is mediated by cell proliferation, because the protein/DNA ratio remains unchanged.

Thus, lung growth enhancement because of vitamin A varies during gestation, becoming more pronounced at E20 when lung growth velocity reaches its peak. At each time point, however, antenatal vitamin A administration attenuates lung hypoplasia to a remarkably similar degree in nitrofen and CDH groups (Fig. 2). In fact, vitamin A administration did not change the percentage difference in lung hypoplasia observed between the nitrofen and CDH groups documented at E20 and E22. These results clearly indicate that vitamin A attenuates lung hypoplasia by interfering with early acting factors.

In CDH groups, vitamin A did not interfere with the ratio of left to right lung weight at any studied time point. As the ratio of left to right lung weight can be considered a direct index of the action of late determinants of lung hypoplasia, this result reinforces our hypothesis that vitamin A cannot restore late mechanically induced lung hypoplasia secondary to thoracic visceral herniation. This conclusion is further supported by the observation that late administration of vitamin A did not affect lung development. We therefore speculate that the benefit from antenatal administration of vitamin A is only because of its direct action on the molecular substrate where the effects of nitrofen are manifest. This could be related to the hypothesis that vitamin A effectively promotes lung budding and bronchial morphogenesis early in gestation, providing the embryonic lung additional potential for growth in subsequent stages of development [18,24].

Our results raise doubts as to whether vitamin A could be beneficial in the treatment of human fetuses with CDH. In clinical practice, the prenatal diagnosis of CDH occurs relatively late in gestation, usually after 16 weeks' gestation (corresponding to E18 of lung development in rat). Although in previous studies vitamin A appeared to be promising for the treatment of lung hypoplasia in CDH, our results with late (E18.5) gestational administration, an equivalent time point to when human fetuses would first be diagnosed with CDH showed no benefit.

In conclusion, in contrast to late determinants of lung hypoplasia, vitamin A appears to act only on the early pathways that ultimately lead to the diaphragmatic defect and pulmonary hypoplasia. This has relevant clinical implications because in human beings, prenatal diagnosis of CDH commonly occurs after 16 weeks' gestation.

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CHAPTER III

HEART MATURITY IN CONGENITAL DIAPHRAGMATIC HERNIA

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Myocardium expression of connexin 43, SERCA2a, and myosin heavy chain isoforms are preserved in nitrofen-induced congenital diaphragmatic hernia rat model

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Index words:
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Connexin 43;
SERCA2a;
Myosin heavy chain isoforms

Abstract

Background: Previous morphological studies had produced controversial results with regard to heart development in congenital diaphragmatic hernia (CDH), whereas a few publications investigated cardiac function and myocardial maturation. Myocardium maturation is associated with age-dependent increasing of gene expression of gap junction protein connexin 43 (Cx43), adenosine triphosphatase of the sarcoplasmic reticulum (SERCA2a), as well as switching of myosin heavy chains (MHCs) from β to α isoforms. Our aim was to evaluate myocardium maturity in nitrofen-induced CDH rat model.

Methods: Fetuses from dated pregnant Sprague-Dawley rats were assigned to 3 experimental groups: control, nitrofen (exposed to nitrofen, without CDH), and CDH (exposed to nitrofen, with CDH). Myocardial samples collected from left ventricle free wall were processed to (i) quantification of messenger RNA (mRNA) of Cx43, SERCA2a, α and β MHC isoforms, as well as β-actin (housekeeping gene); and (ii) separation of MHC isoforms (α and β isoforms) by sodium dodecyl sulfate polyacrylamide gel electrophoresis silver stained.

Results: We demonstrated that there is no difference in myocardial gene expression of Cx43 (control, 1.0 ± 0.1; nitrofen, 1.1 ± 0.2; CDH, 1.3 ± 0.2) and SERCA2a (control, 1.0 ± 0.1; nitrofen, 0.9 ± 0.1; CDH, 1.0 ± 0.2). Myocardial gene expressions of α and β mRNA of MHC isoforms were slightly decreased both in nitrofen and CDH fetuses when compared with control fetuses, but evaluation of the α-

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to-β ratios of MHC isoforms at protein level revealed no significant differences between CDH and control (control, 16.9 $\pm$ 2.5; CDH, 17.0 $\pm$ 2.0).

Conclusions: Myocardial quantification of Cx43 and SERCA2a mRNA, as well as the expression pattern of MHC isoforms at protein levels, was similar in all studied groups. We predict, therefore, that acute heart failure commonly observed in CDH infants might be attributed predominantly to cardiac overload secondary to severe pulmonary hypertension rather than to myocardial immaturity.

Despite improvements in perinatal care, the mortality of fetuses and newborns with congenital diaphragmatic hernia (CDH) remains exceedingly high [1]. This mortality is related to severe pulmonary hypoplasia and pulmonary hypertension [2]. Nevertheless, some authors had suggested that, even in the absence of congenital heart disease, these patients could suffer some cardiac underdevelopment that might further worsen their prognosis [3-5].

Research in experimental CDH had documented that there are 2 determinants of lung hypoplasia [6]: early in gestation, lung underdevelopment seems to be linked to molecular factors, whereas late determinants are related to mechanical compression from herniated organs. On the other hand, heart development in CDH appears to be influenced only by early factors acting during fetal growth [7,8]. In fact, in the nitrofen rat model of CDH, we demonstrated that heart hypoplasia occurs early in gestation, but disappears as gestation proceeds to term, suggesting that late determinants do not influence heart development in CDH [8]. Nevertheless, although morphologically normal at the end of gestation, the heart could be functionally immature. It remains to be clarified, therefore, if myocardium in CDH fetuses is more immature than age-matched controls, as described for lung [2,9].

Systolic and diastolic cardiac function in normal fetuses and newborns has less reserve than in the adults. Studies in experimental models demonstrated that systolic pump performance and contractility are developmentally regulated processes that improve throughout gestation and during postnatal life [10]. Active and passive cardiac relaxation and global diastolic function also improve as a function of perinatal age [11-13]. These changes in cardiac function typically are accomplished by qualitative and/or quantitative alterations in cardiomyocyte-specific gene expression. Such adaptation occurs at different levels either at cell-to-cell communication as well as at intracellular calcium kinetics and contractile proteins.

At the cell-to-cell communication level, connexin 43 (Cx43) is a major constitutive protein of the gap junctions in the mammalian myocardium involved in the electrical coupling of myocytes [14]. Abundance of Cx43 is developmentally regulated. In fact, it was already demonstrated that the extent of Cx43 expression in the ventricles progressively increases during development [15].

At the intracellular level, sarcoplasmic reticulum Ca^{2+}-adenosine triphosphatase (SERCA2a) controls calcium reuptake by the sarcoplasmic reticulum from the cytosol during diastole. During early fetal development, calcium exchanges in ventricular myocardium are mainly controlled by the Na^{+}-Ca^{2+} exchanger of the plasma membrane, whereas SERCA2a plays an important role only at the end of gestation and after birth [16]. Myocardial maturation is accomplished by an increase in transcription of SERCA2a messenger RNA (mRNA) as gestation proceeds to term. Myosin is a hexameric contractile protein, composed of 2 myosin heavy chains (MHCs), which hydrolyses adenosine triphosphate required for cardiac muscle contraction. Mammalian cardiac muscle can express 2 MHC isoforms: α and β. The α isoform (α-MHC) is associated with a higher adenosine triphosphatase activity than the β isoform (β-MHC). The specific pattern of cardiac MHC isoform expression changes during development and strongly influences the contractile properties and energetics of the heart during fetal growth [17-22].

In heart failure and pressure overload fetal models, it was previously demonstrated a dowregulation of Cx43 [23] and SERCA2a [24], reflecting myocardial stress and immaturity. In addition, evaluation of myocardial α-to-β ratio of MHC isoforms has been suggested as a molecular marker of myocardial maturation and adaptation to atrophy, hypertrophy, failure, and hypoplasia.

The aim of the current study was to evaluate, in the nitrofen-induced CDH rat model, gene expression of different cardiac molecular parameters that are developmentally regulated in an attempt to detect any molecular signs of myocardium immaturity.

1. Materials and methods

The protocols used in this investigation were approved by the Institutional Animal Care and Use Committee and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

1.1. Animal model

Seven female Sprague-Dawley rats (225 g; Criffa, SA, Barcelona, Spain) were pregnant after controlled overnight mating, and the finding of vaginal plug was counted as day 0. Pregnant rats were exposed on 9.5 days post coitum to 100 mg of nitrofen (2,4-dichlorophenyl-*p*-nitrophenylether) dissolved in 1 mL of olive oil administered by gavage or with an equal volume of olive oil alone [25]. Fetuses were harvested by cesarean section at day 21.5 (term gestation,

22 days) and weighed on a precision balance (Scaltec Instruments SBC 21, Heiligenstadt, Germany). After fetal decapitation, under binocular surgical microscopy (Leica, Wild M651.MS-D, Heerbrugg, Switzerland), laparotomy was performed to inspect the diaphragm. Lungs and hearts were excised in bloc through median sternotomy. Heart and left lung were weighed, and their wet weights were expressed as a percentage of fetal body weight. Myocardial samples were harvested from left ventricular free wall, weighed, and snap frozen at −80°C for further molecular studies. Fetuses were assigned to 3 experimental groups: (i) *control group*, fetuses exposed to olive oil alone, without CDH; (ii) *nitrofen group*, fetuses exposed to nitrofen without CDH; (iii) *CDH group*, fetuses exposed to nitrofen with CDH. Fetuses with structural cardiac defects were excluded.

1.2. Molecular studies

1.2.1. Ribonucleic acid extraction

Total mRNA from left ventricle sample of 30 fetuses (Control group n = 10, Nitrofen group n = 10, CDH group n = 10) was extracted using the RNeasy Mini Kit Protect (74712; Qiagen, Germany). Quantification of total mRNA was done by spectrophotometry (BioPhotometer; Eppendorf, Germany), and the ratio A260/A280 was used to test protein and deoxyribonucleic contamination of the extracted product.

1.2.2. Reverse transcription

Each sample of mRNA was diluted until it reaches 50 ng/μL; for the complementary DNA (cDNA) transcription reaction, 2 μL was used (100 ng). The reverse transcription was carried out on a T-gradient thermoclycer (Biometra, Germany) using the kit Superscript II (48190-001; Invitrogen). Briefly, 1 μL of 125 ng/μL random primers (48190-001; Invitrogen), 4 μL of 5× buffer, 2 μL of 1 mmol/L 1,4-dithiothreitol (DTT), 1 μL of 10 mmol/L deoxyribonucleotide triphosphate (dNTP) mix (R0192; MBI Fermentas), 1 μL of recombinant Rnasin (N2515; Promega), and 1 μL of Superscript II reverse transcriptase were added in a total volume of 20 μL. The following program was used to carry on the reverse transcriptase reaction: 42°C for 60 minutes and 70°C for 15 minutes. In all the reactions, a negative control, omission of mRNA, was used.

1.2.3. Quantitative polymerase chain reaction

Accordingly to a 2-step model, the obtained total cDNA was used for the relative quantification by real-time polymerase chain reaction (PCR) of SERCA2a, Cx43, α-MHC, β-MHC, and of the reference gene β-actin. The PCR reactions were prepared for the LightCycler (Roche, Germany). Each run for all gene quantification consisted of 15 minutes of hot-start and 55 cycles (95°C for 15 seconds, 58°C for 20 seconds, 72°C for 15 seconds). The following protocol was used for each capillary (11909339001, Roche): 10 μL of SYBR Green (204143, QuantiTech SYBRgreen PCR; Quiagen, Germany), 2 μL of cDNA, and 10 pmol of each primer (Thermo Electron, Germany) were added in a total volume of 20 μL. A negative control was included in all the runs, which consisted in omitting the cDNA and performing a melting curve analysis, thus, allowing the detection of putative contamination.

Primer design was based on the available sequences in GenBank (NCBI-NLM-PubMed-Gene). All the primers are intron spanning (Table 1). For all the primer sets, standard amplification curves were made with randomly selected cDNA samples setting, $r = 0.99$. In every quantitative run, 3 replicas of the standard ST-100 ng were included as internal controls and quantitative references for the variability of the procedure. In all the samples, gene expression was normalized for β-actin.

1.2.4. Myosin heavy chain protein isoforms

We evaluated the content of protein MHC isoforms in control and CDH fetuses. For that, left ventricular samples from 15 fetuses (*control group*, n = 7; *CDH group*, n = 8) were homogenized in a solution: 50 mmol/L N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) (USB 16928), 150 mmol/L KCl (Merck 1.04936), 1 mmol/L phenylmethylsufonyl fluoride (PMSF) (Sigma P-7626), 1 mmol/L DTT (PlusOne 17-1318-02), 0.1 mmol/L CLAP (chemostatin, leupeptin, antipain, pepstatin). The entire protein content of each sample was obtained by spectrophotometry using the Bradford method (BioRad 500-0002). Twenty micrograms of the total protein content from each sample were separated by electrophoresis in 10% stacking and 5% separating sodium dodecyl sulfate polyacrylamide

Table 1 Primers used for quantitative PCR

Gene	Accession no.	Primer set	Product size (base pair)
α-MHC	NM_017239	5′ -GCTTTGGGAAGTTCATCAG-3′ 5′ -GCCTTTAGCTGGAAGATCAC-3′	109
β-MHC	NM_017240	5′ -CTGAGGAGGCGGAGGAACAG-3′ 5′ -CTTGGCGCCAATGTCACG-3′	146
Cx43	NM_012567	5′ -GATTGAAGAGCACGGCAAGG-3′ 5′ -GTGTAGACCGCGCTCAAG-3′	144
SERCA2a	NM_017290	5′ -TGGTCTGTATCTTCCTGACG-3′ 5′ -CCAGATCTGGAGGATTGAAC-3′	129
β-actin	NM_031144	5′ -GAT TTG GCA CCA CAC TTT CTA CA-3′ 5′ -ACT TTG GTC ATC TTT TCA CGG TTG G-3′	114

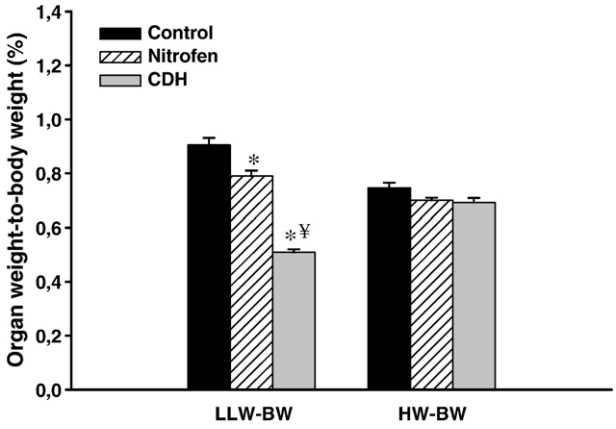

Fig. 1 Left lung-to-body weight (LLW-BW) and heart-to-body weight (HW-BW) ratios in control, nitrofen, and CDH groups at term of gestation ($P < .05$ vs *control group, vs ¥nitrofen group).

gels (60 V, 240 minutes). Subsequently, α and β MHC isoforms were identified by comparison with a molecular weight standard (161-0373 BioRad) after silver staining (BioRad 161-0449) with a continued agitation to obtain a uniform staining across gels. Relative quantification of MHC isoforms was achieved through scanning densitometry (white light; Alpha innotech Alpha Imager 2200).

1.3. Statistical analysis

Results were presented as mean ± SEM. The different data sets of control, nitrofen, and CDH groups failed in the Kolmogorov-Smirnov test for normality. Therefore, statistical analysis was performed by the 1-way analysis of variance on ranks, and the Dunn test for posttest analysis. Statistical significance was set at $P < .05$.

2. Results

From fetuses exposed to nitrofen, 58% had diaphragmatic defect. At term, the left lung-to-body weight ratio was significantly reduced in CDH group in comparison to non-CDH groups. In contrast, the heart-to-body weight ratios were not significantly different (Fig. 1).

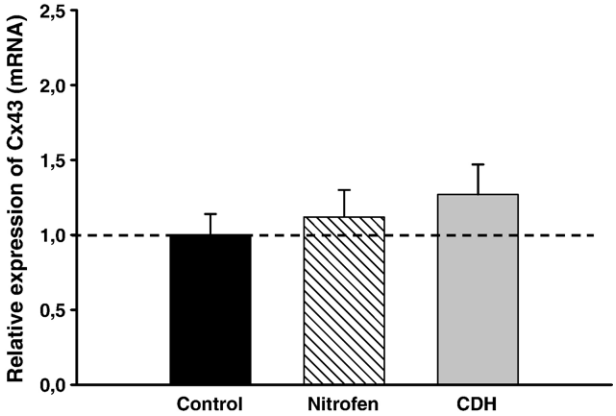

Fig. 2 Expression of Cx43 mRNA in control, nitrofen, and CDH groups expressed in arbitrary units normalized for β-actin.

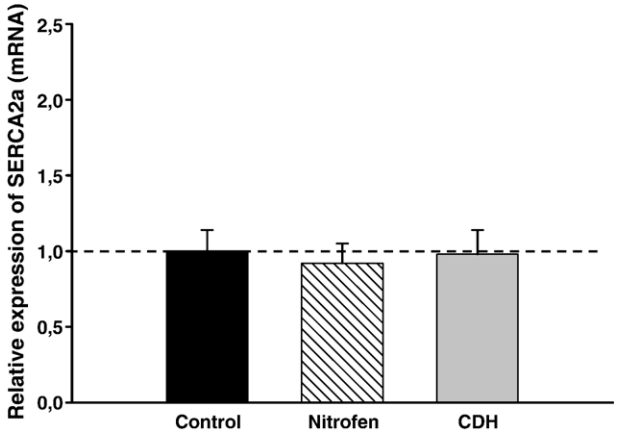

Fig. 3 Expression of SERCA2a mRNA in control, nitrofen, and CDH groups expressed in arbitrary units normalized for β-actin.

The levels of mRNA (normalized to β-actin) of both Cx43 (Fig. 2) and SERCA2a (Fig. 3) were similar between all studied groups. Regarding MHC isoforms, we demonstrated

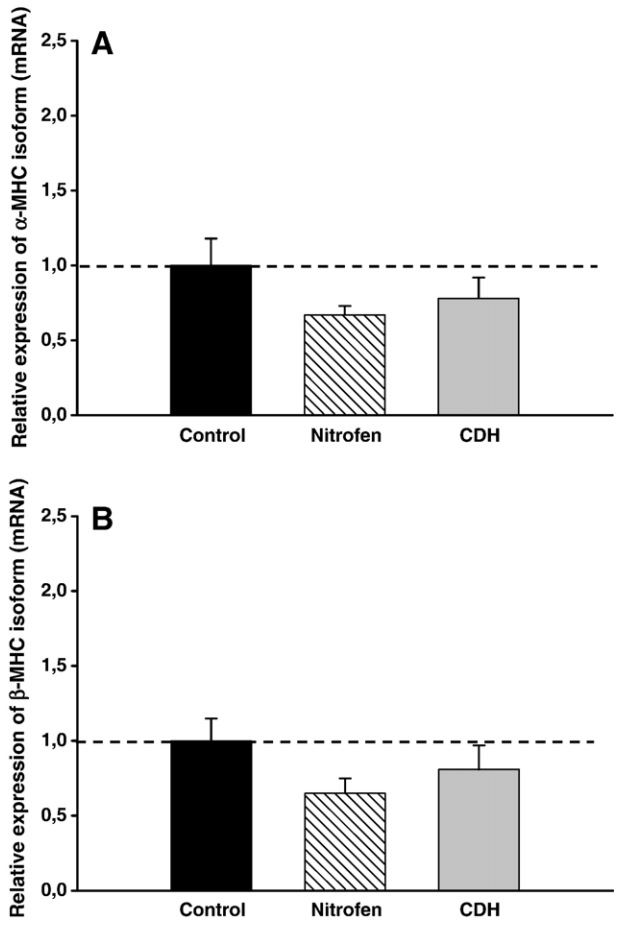

Fig. 4 Expression of α (A) and β (B) MHC isoforms mRNA in control, nitrofen, and CDH groups expressed in arbitrary units normalized for β-actin.

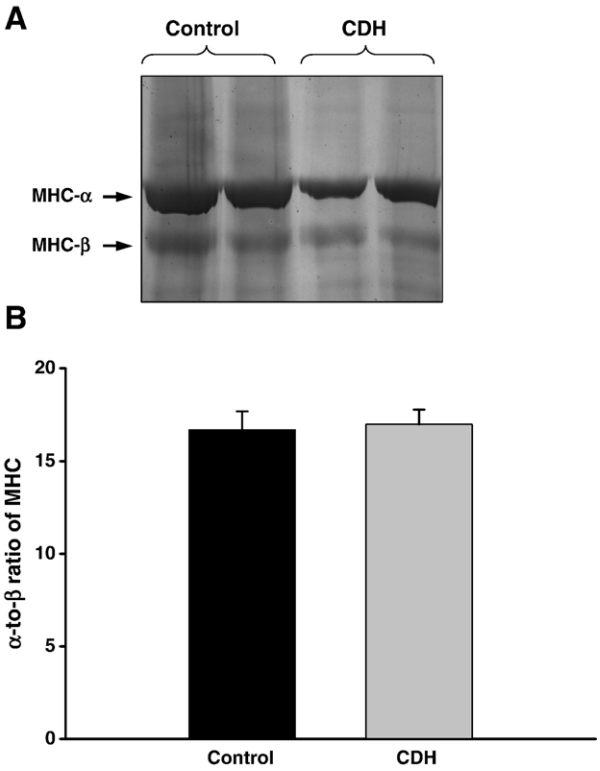

Fig. 5 A, Representative gel with separation of α and β MHC isoforms in control and CDH groups. B, α-to-β ratios of MHC at protein level.

that myocardial gene expressions of α and β mRNA of MHC isoforms were slightly decreased both in nitrofen and CDH fetuses when compared with control fetuses (Fig. 4).

To confirm these results, we performed additional studies to evaluate α-to-β ratios of MHC isoforms at protein level. Despite similar molecular masses of rat protein α-MHC and β-MHC (~223 kd, with difference <0.2%), we achieved consistent separation of both protein isoforms from small samples of myocardium with selected electrophoresis protocol. In separating gels applied, β-MHC migrated farther than α-MHC, as expected (Fig. 5A). Our results demonstrated that control fetal rat's left ventricle expresses predominantly α-MHC protein isoform with only a small amount of β-MHC, and this pattern is similar in fetuses with CDH (α-MHC control, 94% ± 0.6; CDH, 95% ± 0.6). Regarding MHC α-to-β ratio, we verified that this parameter of molecular adaptation did not change in fetuses with diaphragmatic defect, because there is no statistical difference in comparison to control group (Fig. 5B).

3. Discussion

Congenital diaphragmatic hernia is a rare (1:2500) congenital malformation with a mortality that remains exceedingly high regardless of sophisticated management techniques, such as extracorporeal membrane oxygenation and fetal tracheal occlusion [1,26]. The leading causes of mortality in CDH are pulmonary hypoplasia and severe persistent pulmonary hypertension, but the presence of chromosomal anomalies and/or congenital heart disease worsens the prognosis of these patients [5].

The link between the heart and lung development in CDH was emphasized by several authors [3-5,8]. In fact, CDH is a complex disease, probably related to an early imbalance in retinoid signaling that occurs in a critical temporal window, interfering not only with diaphragmatic closure, but also with the development of several organs such as lung and heart [27,28]. In this sequence, it has been suggested that even the fetuses that do not present heart malformations could have some type of cardiac molecular changes. In previous studies, some investigators have reported significant heart hypoplasia [29] and structural immaturity of the heart in rat fetuses with CDH [9]. In the CDH rat model, the dual-hit hypothesis defines that there are 2 determinants of lung hypoplasia: early determinants are related to molecular factors likely secondary to disturbances in retinoid signaling, whereas late determinants are related to mechanical compression secondary to organ herniation [6-8]. We previously demonstrated that during fetal development, the heart is only affected by early determinants. In fact, early in gestation, there is evidence of heart and lung hypoplasia, but as gestation proceeds to term, lung hypoplasia increases, whereas heart hypoplasia recovers to normal [8]. Interestingly, this recovery seems to occur also in humans. In fact, during fetal life, the echocardiographic evaluation of left ventricular size was suggested to be useful in predicting the outcome of fetuses with CDH [4,5,30], whereas at end gestation, recent studies failed to document significant heart hypoplasia and usefulness in estimation of left ventricular mass to predict the outcome in infants with CDH [31,32]. Even in these conditions, it has been suggested by some authors that the left ventricle at delivery in CDH infants could be less compliant and less able to maintain the systemic circulation. In this sequence, we hypothesized that left-ventricular myocardium could be functionally immature, although morphologically normal at the end of gestation.

Evaluation of cardiac function in rat newborns is hardly performed because of reduced dimensions of pups. To overcome this limitation, we selected some genes that are developmentally regulated and sensible to load conditions. For these reasons, we evaluated myocardial maturity through gene expression of Cx43, SERCA2a as well as MHC isoforms.

It is important to highlight that in our study, we did not include fetuses with cardiac congenital malformations because congenital heart disease can modify the global heart weight as well as molecular parameters of maturity. In addition, as molecular parameters of maturity could be affected by toxic effect of nitrofen, we included in our study fetuses exposed to nitrofen without CDH.

Connexin 43 is important for myocardial communication through gap junction, which is essential for myocardial electrical activity, heart differentiation, and function since the early stages of heart morphogenesis [14,15]. SERCA2a is a sarcoplasmic pump involved in calcium reuptake that regulates either inotropic or lusitropic reserve of the fetal heart [11,16]. Interestingly, we could not detect significant changes on myocardial expression of Cx43 and SERCA2a neither in nitrofen nor in CDH groups. Based on these results, we predict no significant functional myocardial disturbances owing to electrical or calcium kinetics issues in the nitrofen-exposed fetuses.

Regarding contractile proteins, myocardial α-to-β ratio of MHC isoforms has been used as an appropriate index to predict both cardiac maturation and dysfunction [18-21]. In fact, the ratio of MHC isoforms changes during fetal development as heart maturation occurs [20], whereas in cardiac dysfunction (both in experimental models and in human disease), it was demonstrated as overexpression of β-MHC isoform [33,34]. Myosin heavy chain isoforms expression has been suggested, therefore, as a molecular marker either of myocardium maturation or of adaptation to atrophy, hypertrophy, failure, and hypoplasia [18-21]. In our study, myocardial gene expressions of α and β mRNA of MHC isoforms were slightly decreased both in nitrofen and CDH fetuses, but evaluation of the α-to-β ratios of MHC isoforms at protein level revealed no significant differences between CDH and control. This suggests that CDH hearts were not associated with myosin isoform immaturity in comparison with age-matched controls. Interestingly, Okazaki et al [35] also studied the expression of myosin isoforms in pulmonary vasculature of CDH rats, and their results in the lung were similar to ours in the heart. Pulmonary vasculature myosin isoform SMem is predominantly expressed in immature smooth muscle cells, whereas SM2 isoform is expressed in mature smooth muscle cells. The authors found that, in the rat model of CDH, there is no difference in pulmonary smooth muscle cell differentiation in CDH fetuses: in both control and CDH groups, SMemb expression was positive from 16 days' gestation, whereas SM2 expression was negative in vessel walls during the prenatal life.

In our point of view, these results provide also additional molecular evidence to reinforce morphological data, which established that no significant left ventricular hypoplasia occurs in CDH fetuses at end gestation [7,8]. In fact, it was suggested that the major pathophysiologic mechanism of the relative underdevelopment of the left side of the heart in CDH is probably an unloading of the left ventricle owing to a combination of decreased pulmonary venous return because of diminished lung mass and a decreased interatrial shunt [30]. In adult rats, Depre et al [36] demonstrated that chronic unloading could reactivate the molecular fetal phenotype of myocardium (increase of β MHC isoform). It seems reasonable, therefore, to hypothesize that if left ventricular hypoplasia would occur secondary to the abovementioned mechanism (unloading), left ventricular myocardium would present delayed maturation, which we could not demonstrate.

In conclusion, we demonstrated in the nitrofen-induced CDH rat model that at term of gestation, there is no evidence of myocardial immaturity. In this sequence, we predict that acute neonatal heart failure commonly observed in CDH infants might be attributed predominantly to cardiac overload secondary to severe pulmonary hypertension rather than to myocardial intrinsic disturbances.

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CHAPTER IV

PERINATAL HEART OVERLOAD ASSOCIATED WITH PULMONARY HYPERTENSION IN CONGENITAL DIAPHRAGMATIC HERNIA

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Perinatal profile of ventricular overload markers in congenital diaphragmatic hernia

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Pulmonary hypertension;
B-type natriuretic peptide;
Angiotensinogen;
Endothelin 1

Abstract

Background: In congenital diaphragmatic hernia (CDH), pulmonary hypertension increases right ventricle (RV) afterload, which could impair heart function and contribute to poor outcome for most affected infants. Nevertheless, the real significance of vascular pulmonary alterations in perinatal hemodynamics is largely unknown. It is defined that ventricular pressure overload induces increased myocardium gene expression of B-type natriuretic peptide (BNP) and components of the renin-angiotensinogen and endothelin (ET)–1 systems. Our aim was to evaluate perinatal myocardium expression of these genes associated with ventricular pressure overload in a nitrofen-induced CDH rat model.

Methods: In the nitrofen-induced CDH rat model, fetuses from dated pregnant Sprague-Dawley rats at 15.5, 17.5, 19.5 and 21.5 days postcoitum as well as newborn pups were assigned to 3 experimental groups: control, nitrofen (exposed to nitrofen, without CDH), and CDH (exposed to nitrofen, with CDH). Myocardial samples collected from the RV and left ventricle (LV) were processed for quantification of messenger RNA (mRNA) of BNP, angiotensinogen, and ET-1.

Results: The perinatal expression of BNP, angiotensinogen, and ET-1 mRNA in the RV and ~~left ventricle~~ of the control group revealed daily changes. During gestation, the expression of BNP and angiotensinogen mRNA underwent significant oscillation compared with control in both nitrofen-exposed fetuses, although we cannot identify significant differences between the nitrofen and CDH groups. After birth, we found a significant increasing expression of all studied genes only in the RV of CDH pups.

Conclusions: Perinatal myocardial quantification of BNP, angiotensinogen, and ET-1 mRNA levels suggests that both nitrofen-exposed and control pups revealed prenatal variations of expression of the studied genes. Moreover, CDH is associated with significant molecular alterations only in the RV after birth.

Despite improvements in perinatal care, the mortality of fetuses and newborns with congenital diaphragmatic hernia (CDH) remains exceedingly high [1]. This mortality seems to be related with severe pulmonary hypoplasia and pulmonary hypertension (PH) [2]. Several authors suggested that, even

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in the absence of congenital heart disease, these infants could experience of some degree of cardiac underdevelopment [3,4]. In previous studies, we demonstrated in the experimental rat model of CDH the absence of left ventricle (LV) hypoplasia [5,6] or myocardium immaturity [7] in CDH fetuses. Nevertheless, the real significance of increased right ventricle (RV) afterload, because of neonatal PH, in heart function is still unknown.

Another question that still persists in CDH is the true significance of PH in fetal hemodynamics and its consequences for the fetal heart. In infants with CDH, pulmonary vascular modifications occur from early stages of prenatal development [8] and assume obvious significance after birth. Nevertheless, their meaning in fetal heart function is uncertain. Because fetal heart function is not easily assessed by ultrasonography, the evaluation of markers of ventricular overload in an experimental model of CDH might provide an appropriate alternative. Several biochemical and genetic markers have been suggested to evaluate ventricular load and function, both in animal models and humans, such as B-type natriuretic peptide (BNP), components of the renin-angiotensin system, and endothelin (ET)–1[9].

B-type natriuretic peptide is a hormone of predominantly ventricular origin produced and released in response to increased ventricular wall stress [10,11]. In recent years, it has emerged as a very sensitive biochemical marker for ventricular dysfunction in heart failure as well as in PH, and its plasmatic level could be used to guide the response to therapy and to predict prognosis [12,13]. During normal fetal rat development, a very intense expression of BNP in the heart from 9.5 days postcoitum (dpc) was demonstrated, with major peaks of expression in stages that coincide with landmarks in heart development [14].

The components of the renin-angiotensin system and their roles in adult cardiac hypertrophy have been well documented [15,16]. In adult hearts, the increased hemodynamic load results in increased levels of angiotensin II that stimulates significant hypertrophy and remodeling of cardiac structure. Recent evidence from in vivo studies indicates that angiotensin II also acts as a growth factor and has a potential role in embryonic, fetal, and neonatal development of the heart [17-19]. In addition, the AT1 and AT2 angiotensin II receptor subtypes are present in the heart and are developmentally regulated [20].

Endothelin 1 is a potent vasoconstrictor peptide derived from endothelial cells that is also produced by cardiac myocytes [21]. Endothelin 1 induces myocardial cell hypertrophy and has potent positive inotropic and chronotropic effects on isolated heart muscle. These actions are mediated by the receptors for ET-1 (ETA and ETB receptors) on the cardiac myocytes [22]. The production of ET-1 in the heart is increased in pressure overload conditions, such as PH [23]. Several studies demonstrated that ET system has an important role in the developing heart, contributing to the formation of anatomical structures such as heart outflow tract and great vessels [24].

Interestingly, it was suggested in several previous studies that natriuretic peptides, angiotensin II, and ET-1 could play a significant role in the PH associated with CDH [25-29]. Moreover, in the rat model of CDH, several authors reported significant modifications in heart expression of components from all these systems. Nevertheless, none of these studies assessed the messenger RNA (mRNA) expression of all these genes in both ventricles throughout gestation [30-32].

The aim of the current study was to evaluate, in the nitrofen-induced CDH rat model, LV and RV mRNA expression of BNP, angiotensinogen, and ET-1, genes previously defined as ventricular overload markers, in an attempt to evaluate the significance of PH in myocardium molecular adaptation during fetal development and transition after birth.

1. Materials and methods

The protocols used in this investigation were approved by the Institutional Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996).

1.1. Animal model

Pregnancy was obtained in 22 female Sprague-Dawley rats (225 g; Criffa, SA, Barcelona, Spain) after controlled overnight mating, and the finding of vaginal plug was counted as day 0. At 9.5 dpc, pregnant rats were exposed to 100 mg of nitrofen (2,4-dichlorophenyl-p-nitrophenylether) dissolved in 1 mL of olive oil administered by gavage or to an equal volume of olive oil alone [33]. Randomly, fetuses, treated with nitrofen or olive oil, were harvested by cesarean section at 15.5, 17.5, 19.5, and 21.5 dpc (term gestation, 22 days), weighed on a precision balance (SBC 21; Scaltec Instruments, Heeligeesdadt, Germany), and killed by decapitation. To evaluate newborn rats, the gestation was continued in some pregnant rats until 22 dpc, and rats were allowed to deliver spontaneously. Newborns rats were harvested immediately after death or electively killed, by decapitation, at 6 hours after delivery. In this regard, it might be emphasized that all CDH pups died before our set point. After weighing pups, laparotomy was performed under binocular surgical microscopy (Wild M651.MS-D; Leica, Herbrugg, Switzerland) to inspect the diaphragm. Hearts were excised en bloc through median sternotomy. Myocardial samples were harvested from LV and RV free wall and snap-frozen at −80° for further molecular studies. Fetuses were assigned to the following 3 experimental groups: (i) control group—fetuses or pups exposed to olive oil alone, without CDH; (ii) nitrofen group—fetuses or pups exposed to nitrofen without CDH; and (iii) CDH group—fetuses or pups exposed to nitrofen with CDH. Fetuses and pups with

structural cardiac defects were excluded. Because it was not feasible to accurately identify a diaphragmatic defect at 15.5 dpc, only 2 groups were defined: control and nitrofen (exposed to nitrofen with or without CDH).

1.2. Molecular studies

1.2.1. Ribonucleic acid extraction and reverse transcription

Total mRNA from LV and RV samples of 150 fetuses (n = 10 each for control, nitrofen, and CDH, in each studied time-point: 15.5 dpc, 17.5 dpc, 19.5 dpc, 21.5 dpc, and newborn) was extracted using the RNeasy Mini Kit Protect (74712; Qiagen, Germany). Quantification of total mRNA was done by spectrophotometry (BioPhotometer, Eppendorf, Germany), and the A260/A280 ratio was used to test protein and deoxyribonucleic contamination of the extracted product.

Reverse transcription was performed as previously described by Santos et al [34].

1.2.2. Quantitative polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed as previously described by Santos et al [34].

Primer design was based on the available sequences in GenBank (NCBI-NLM-PubMed-Gene). All the primers are intron spanning (Table 1). For all the primer sets, standard amplification curves (ST curves) were made with randomly selected complementary DNA samples, setting $r = 0.99$. ~~In every quantitative run, 3 replicas of the standard ST-100 ng were included as internal controls and quantitative references for the variability of the procedure.~~ The samples gene's expression was normalized for β-actin.

1.3. Statistical analysis

Results were presented as mean ± SEM. Statistical analysis was performed using the SigmaStat 3.5 software. The different data sets of control, nitrofen, and CDH groups failed in the Kolmogorov-Smirnov test for normality. Therefore, statistical analysis was performed by the 1-way analysis of variance on ranks and the Dunn test for posttest analysis. Statistical significance was set at $P < .05$.

2. Results

The mRNA levels of BNP, angiotensinogen, and ET-1 (through gestation, in the RV and LV, in the control, nitrofen, and CDH groups) are presented in Figs. 1–3, respectively.

The BNP mRNA expression in the RV demonstrated in control group a significant decrease of around 19.5 dpc, followed by a peak increase at 21.5 dpc. After birth, its levels are similar to earlier studied stages of heart development. In comparison with the control group, we found significant differences both in nitrofen and CDH groups, characterized by a mirror-image pattern of expression, with inverse peak increase at 19.5 dpc and a subsequent decrease at 21.5 dpc. After birth, the BNP mRNA expression in RV was similar in the nitrofen and control groups, but in the CDH group, we found a significant increase in its expression (5.5-fold) (Fig. 1).

The BNP mRNA expression in LV revealed a similar expression pattern in all study groups during fetal development. The control group had a significant decrease of around 19.5 dpc followed by an increase at 21.5 dpc, although its magnitude was not as evident as occurred in the RV. Regarding the nitrofen and CDH groups, we also found a mirror-image pattern of expression. Nevertheless, after birth, the levels of BNP mRNA were similar to prior stages of heart development in the 3 groups (Fig. 1).

Concerning the expression of angiotensinogen mRNA in the RV, we observed a slight increase from 17.5 dpc that peaked at 19.5 dpc, followed by a decrease to basal levels at 21.5 dpc. After birth, its levels remained constant. In the nitrofen and CDH groups, there was no significant variation in angiotensinogen mRNA expression through gestation. At birth, a significant rise occurred in the CDH group, compared with the control and nitrofen groups (Fig. 2).

Similar to the expression of BNP, in the control group, we found an LV angiotensinogen mRNA expression pattern similar to RV. Its expression rose from 17.5 dpc, peaked at 19.5 dpc, then slowly decreased until birth. Regarding the nitrofen and CDH groups, angiotensinogen mRNA levels in LV did not vary during gestation and after birth (Fig. 2).

In the control group, ET-1 mRNA level in RV showed a significant peak of expression at 19.5 dpc, followed by a constant level of expression that persisted even after birth. During fetal development, both the nitrofen and CDH groups

Table 1 Primers used for quantitative polymerase chain reaction

Gene	Accession no.	Primer set	Product size (base pair)
BNP	NM_031545	5′-GCA GAA GCT GCT GGA G-3′ 5′-GCT GTC TTG AGA CCT AAG GA-3′	118
Angiotensinogen	NM_134432	5′-GGATAAGTCCAGAGAGCGAG-3′ 5′-CAGACACCCCTGCTACAGTC-3′	129
ET-1	NM_012548	5′-CAGAAACAGCTGTCTTGGGA-3′ 5′-GGAGGAGCAGGAGCAACG-3′	116
β-actin	NM_031144	5′-GAT TTG GCA CCA CAC TTT CTA CA-3′ 5′-ACT TTG GTC ATC TTT TCA CGG TTG G-3′	114

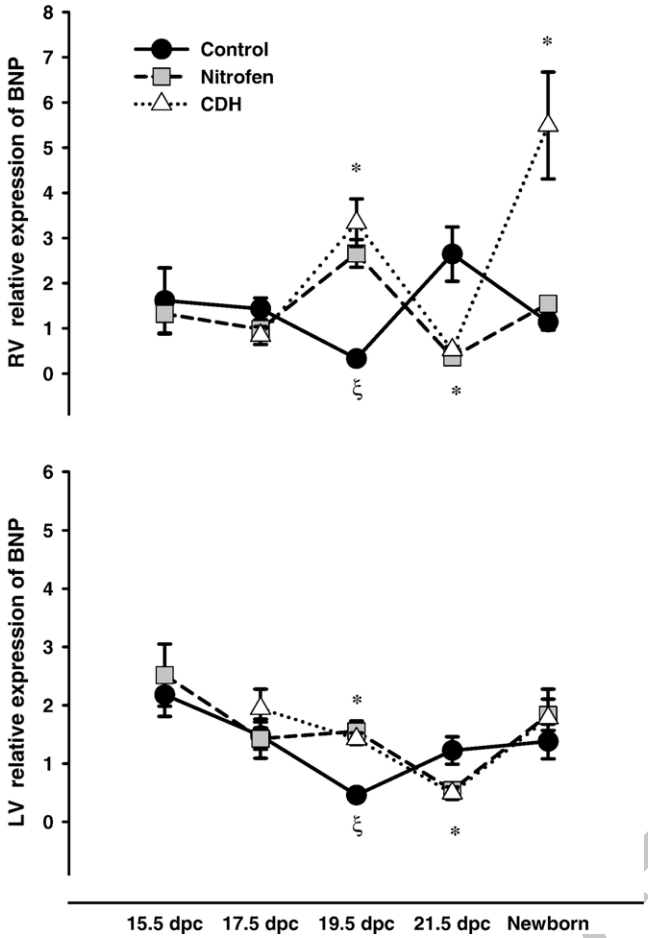

Fig. 1 Expression of BNP mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ vs 16 dpc.

had exactly the same expression pattern, without any significant difference from control. However, after birth, whereas in the nitrofen group, we did not found any difference from the control group, the CDH group had a significant increase in ET-1 mRNA expression (Fig. 3).

Finally, the LV the expression of ET-1 mRNA showed a peak at 21.5 dpc and returned to baseline levels after birth. In the nitrofen and CDH groups, we found the same pattern of expression of the gene, without any difference compared with the control group (Fig. 3).

To evaluate the relative expression changes of mRNA of studied genes in the LV and RV after birth, we used the RV-to-LV mRNA ratio (Fig. 4). In the control group, mRNA of the 3 genes had similar relative expression in both ventricles. On the other hand, in the CDH group, we found a very significant increase in the RV-to-LV ratio expression patterns of BNP, angiotensinogen, and ET-1.

3. Discussion

In this study, we determined the cardiac expression of BNP, angiotensinogen, and ET-1 mRNA during perinatal development in normal and nitrofen-exposed rats. Cardiac expression of these genes showed temporal changes, suggesting a closely regulated developmental expression. Our results also showed late fetal cardiovascular disturbances in nitrofen-exposed fetuses. Moreover, it clearly illustrates that CDH pups, in early postnatal adaptation, experience severe RV molecular adaptation to pressure overload.

In our day, CDH remains a challenge in perinatology. The most severely affected babies have an extremely high mortality rate despite aggressive treatment. Persistent hypoxia because of pulmonary hypoplasia and hypertension is considered by several authors as the main problem in CDH. Nevertheless, centers without extracorporeal membrane oxygenation did not significantly improve the outcome using techniques targeted to lung-dependent oxygenation, such as high-frequency ventilation or pulmonary vasodilator therapy. Although the real significance of PH is not completely defined in infants with CDH, it is well known that severe PH is associated with increased mortality

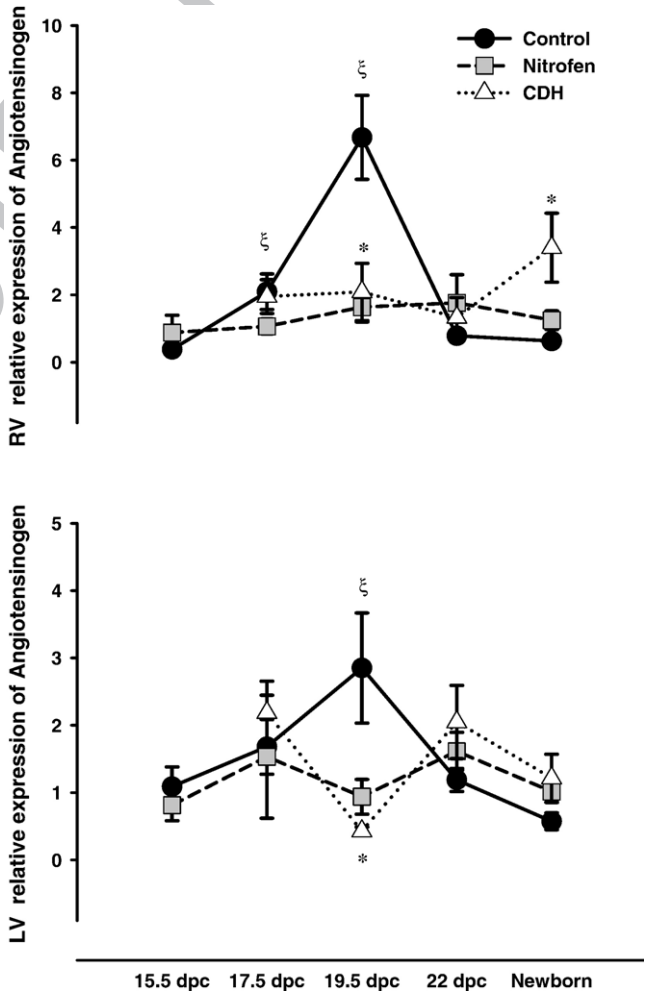

Fig. 2 Expression of angiotensinogen mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ 16 dpc.

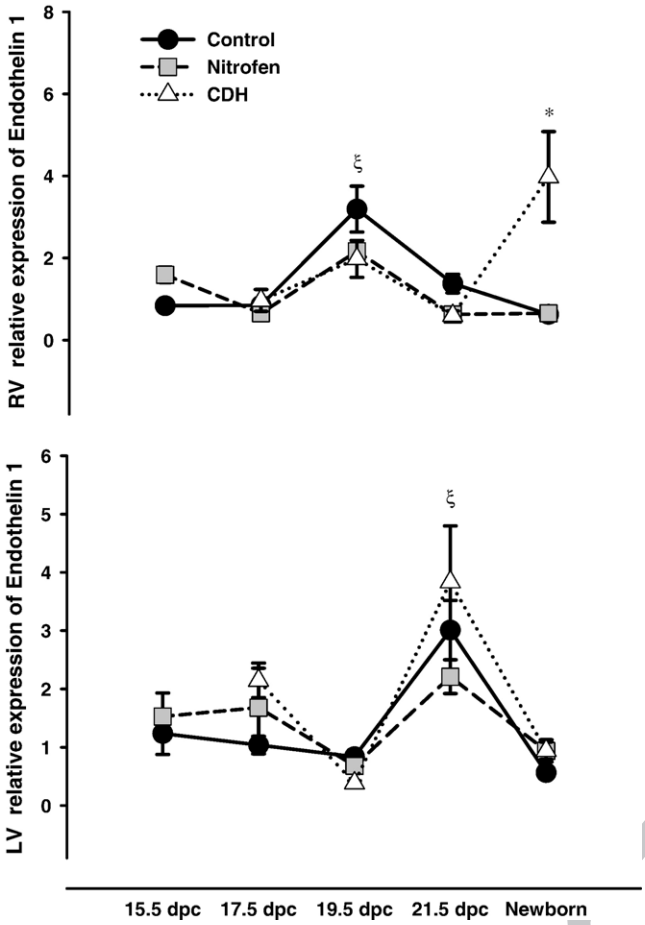

Fig. 3 Expression of ET-1 mRNA from 15.5 dpc to 6 hours of postnatal life in control, nitrofen, and CDH fetuses, both in RV (upper) and LV (bottom). *$P < .05$ vs control group; $^{\xi}P < .05$ vs 16 dpc.

[35]. Pulmonary hypertension interferes with gas exchange as well as hampers myocardial performance, with additional compromise of pulmonary blood flow and tissue oxygenation [36].

From fetal to adult life, cardiopulmonary interaction plays a significant role in physiologic hemodynamics. Pathologic conditions affecting the lung or the heart could hamper this equilibrium. Although the pulmonary consequences of cardiac disease are well recognized, the influences of pulmonary changes on cardiac function are less well appreciated, particularly in fetuses. In late fetal life, several physiologic hemodynamic changes occur, such as initiation of ductus arteriosus closure and pulmonary vasodilatation, as well as increase in RV output to the pulmonary artery [37]. These modifications are promoted by many paracrine factors, among which systems such as natriuretic peptides, angiotensin, and ET-1 are supposed to play a role in different stages of heart development. Although significant alterations in these systems are well documented in pressure and volume cardiac overload in adults, the exact expression pattern of these genes in relation to fetal cardiac load was not previously defined. In our study, we demonstrated a distinctive cardiac expression pattern of these genes during normal perinatal fetal rat development, presumably related to hemodynamic variations.

Endothelin 1 mRNA expression is different in the LV and RV, probably because of different loads in the LV and RV. This gene has a peak expression at 19.5 dpc in the RV, whereas in the LV, this occurs at 21.5 dpc. On the other hand, angiotensinogen and BNP have a very similar expression pattern in the RV and LV but a mirror expression compared with each other. We established that, in both ventricles, when BNP increases, angiotensinogen decreases. The opposed effect of natriuretic peptides and angiotensin in myocardium as well as in vessels is well known. Generally, BNP has potent inhibitory effects on the renin-angiotensin-aldosterone system [38], and in heart failure, activation of the renin-angiotensin-aldosterone system is suppressed by BNP [39]. These systems might form an important regulatory complex of fetal vascular physiology and development. Hypothetically, dysregulation of these delicate control mechanisms could interfere with the cardiopulmonary hemodynamics and lead to disease.

Pulmonary vascular abnormalities in CDH have been well described from early stages of lung development. They consist of fewer pulmonary arteries per unit lung volume and peripheral muscularization of small arteries with medial and adventitial thickening [8]. Although the underlying mechanisms are not completely understood, these anomalies may cause abnormal vascular reactivity, and CDH lungs may become unable to adapt normally at birth [40]. The importance of abnormal vascular development as a determinant of survival in CDH has just recently been appreciated. Nonetheless, we are far from understanding the specific interplay of the factors regulating vascular tone in CDH as well as the significance of those anomalies during fetal heart and lung development.

In our work, we intended to define the fetal and neonatal expression pattern of 3 genes related to ventricular pressure

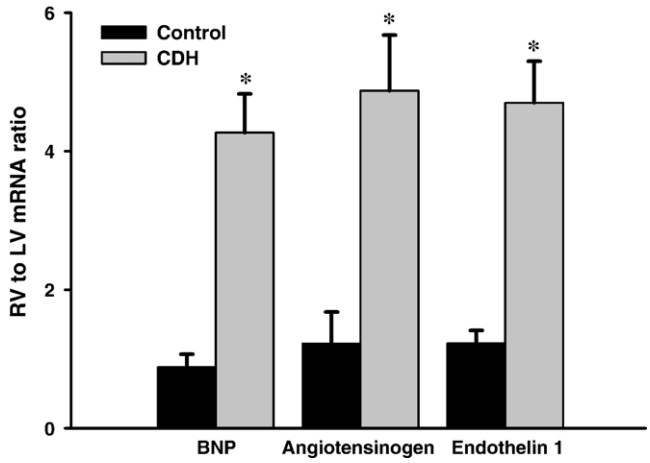

Fig. 4 Right ventricle–to–LV mRNA ratio of BNP, angiotensinogen, and ET-1 in control and CDH groups at 6 hours after birth. *$P < .05$ vs control group.

load in the nitrofen rat model of CDH. We demonstrated that during late fetal life, the nitrofen-exposed fetuses had significant variations in heart expression of mRNA BNP and angiotensinogen, evocative of cardiovascular disturbances. Remarkably, the mirror expression pattern of these 2 genes observed in control rat fetuses is preserved in nitrofen-exposed fetuses. Nevertheless, there are no alterations in ET-1 mRNA cardiac expression, and the variation reported in angiotensinogen and BNP genes occurred both in CDH and non-CDH nitrofen–exposed fetuses. These results suggest that the abnormalities observed are probably consequence of nitrofen action and not related to a hypothetical pulmonary vascular remodeling. We believe that, as occurs in several congenital malformations, PH in CDH is balanced during fetal life and should not have hemodynamic consequences or induce cardiac adaptation. In fact, pulmonary vascular remodeling, when present, should not cause elevated RV pressures in the fetus given the presence of the ductus arteriosus.

In infants with CDH, intrauterine pulmonary hypoplasia and vascular remodeling may cause failure of pulmonary vascular resistance to fall at birth. This event implies an increased pressure overload to RV, with additional wall and endothelium stress, responsible for the initiation of cardiac adaptation. Several studies suggest that in PH, the LV experiences both systolic and diastolic function adaptation because of bulging of the ventricular septum and diminished RV output [41]. In our study, the increased levels of all studied cardiac pressure overload markers in the RV of CDH pups after birth may indicate increased pulmonary vascular resistance in the CDH group. This response is specific for RV, and in this model, we did not demonstrate any LV molecular adaptation to PH.

In conclusion, perinatal myocardial quantification of BNP, ET-1, and angiotensinogen mRNA demonstrated that CDH is associated with significant molecular adaptation only in RV after birth. In fact, although nitrofen induced a hemodynamic imbalance in the expression of these genes, the major and novel observation from our work is the absence of cardiac impact of PH during late fetal life.

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CHAPTER V

CORRELATION OF NT-proBNP AND RIGHT VENTRICLE PRESSURE

Brain-Type Natriuretic Peptide in the Diagnosis and Management of Persistent Pulmonary Hypertension of the Newborn

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Pediatrics 2005;115;1111-
DOI: 10.1542/peds.2004-2780

and to compare our findings with those of Miele et al, which used similar diagnostic criteria and study design. I believe that examining differences in the clinical course of infantile regurgitation between different ethnic groups as mentioned here will be beneficial for understanding the nature of GER in children in general.

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doi:10.1542/peds.2004-2504

In Reply.—

We thank Dr Osatakul for his thoughtful comments. By the comparison of our and his data, it seems that the natural course of infant regurgitation, diagnosed on the basis of the Rome diagnostic criteria,[1] in Italian infants is slightly different from that in Thai subjects. In fact, this functional disorder seems to persist in Italian subjects for a longer time during the 1-year follow-up despite a larger use of antiregurgitation drugs. The reasons for these differences are not known, but they may indicate both genetic and environmental factors peculiar to these particular racial groups. As suggested by Osatakul et al,[2] the variation in gastroesophageal reflux (GER)-related genomes could play a role in the differences in epidemiology among different groups. Recent reports have suggested that both pediatric and adult-onset GER have major genetic components.[3,4] In a recent study, a clear relationship between maternal symptoms of GER and symptoms of spilling in infancy and middle-childhood GER has been found, suggesting that genetic factors are important in milder GER.[4] In addition, in adult Chinese subjects, it has been demonstrated that most of the factors involved in the pathogenesis of GER disease, previously described in Western studies, are present but at lower scale.[5] The acidity of gastric contents is reduced spontaneously or by *Helicobacter pylori* infection. A low-fat diet contributes to a more favorable gastric distribution of the meals and reduced obesity and lowers the number of transient lower esophageal relaxations. The prevalence of hiatal hernia is low and esophageal motility disorders are moderate. Some of these factors could be responsible for the differences in the prevalence and in the natural course of the infant regurgitation in Western and non-Western infants as well. In conclusion, the role of several interesting and important factors needs to be clarified with additional cross-cultural genetic and environmental studies.

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Brain-Type Natriuretic Peptide in the Diagnosis and Management of Persistent Pulmonary Hypertension of the Newborn

To the Editor.—

We read with great interest "Brain-Type Natriuretic Peptide in the Diagnosis and Management of Persistent Pulmonary Hypertension of the Newborn" by Reynolds et al.[1] The authors suggest the blood level of brain-type natriuretic peptide (BNP) as a marker that can be used as an adjunct parameter to differentiate newborns with persistent pulmonary hypertension from other noncardiac causes of respiratory distress. This information might be particularly useful in deciding appropriate treatment and planning early transfer to centers with echocardiographic facilities. Although hypothesized in their discussion, the study by Reynolds et al did not investigate if cardiac anomalies resulting in ventricular stress in neonates might also induce elevated blood levels of BNP. It is worth clarifying this aspect, because these patients could require a different therapeutic approach than infants with persistent pulmonary hypertension.

It is interesting to note that we currently are performing a clinical study aimed at assessing the clinical value of BNP measurements in newborns with congenital heart disease that results in pressure overload to the right ventricle. In this setting, we already studied 7 newborns that required catheterization with therapeutic purpose (1 with pulmonary atresia with intact ventricular septum and 6 with critical pulmonary stenosis). None of them had other morphologic or chromosomal anomalies. All the newborns revealed echocardiographic signs of right ventricular pressure overload, with significant tricuspid regurgitation, right-to-left interauricular shunting, and high estimated right ventricular pressure/mean blood pressure ratio (>2:3). Measurement of BNP level was performed in venous blood samples collected from femoral vein before contrast injection. None of these newborns were submitted to cardiac catheterization without a clinical purpose, and no additional blood was required. In this preliminary study, we found a significantly high value of BNP (>2500 pg/mL) in all patients. Additionally, we found a positive and significant correlation between BNP levels and systolic right ventricular pressure ($r = 0.81$; $P < .05$) as well as to the mean right ventricular pressure ($r = 0.77$; $P < .05$).

In conclusion, our preliminary results confirm the suspicion from Reynolds et al that cardiac anomalies such as pulmonary stenosis/atresia might also result in elevated blood levels of BNP, which correlated with right ventricular pressures. We believe, therefore, that the blood level of BNP reflects ventricular stress, and its elevation should be a criterion for prompt additional evaluation but not necessarily an indication for beginning any specific therapy directed at persistent pulmonary hypertension.

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doi:10.1542/peds.2004-2780

In Reply.—

I thank Baptista et al for their interest in my research. In the article, my co-workers and I[1] hypothesized that brain-type natriuretic peptide (BNP) levels would be elevated in any pathologic condition that resulted in abnormal stress on the ventricles. This would be true of some, but not all, forms of congenital heart disease. It seems from the preliminary data that the doctors are on their way to confirming that hypothesis.

We excluded infants with congenital heart disease from our study because of potential limitations of using the BNP assay in this setting that occurred to us while planning for this project. First, BNP is a marker for ventricular stress. It is not specific to any particular disease state. Although BNP is elevated in infants with persistent pulmonary hypertension (PPHN), it can also be expected to be elevated in any infant with congenital heart disease that results in a state of ventricular stress. Second, low levels of BNP, while suggestive of no ventricular stress, do not rule out serious (potentially life-threatening) cardiac or respiratory diseases.

As we concluded in the article, "the results of this study should not be interpreted as showing that BNP levels are diagnostic of PPHN or that measuring BNP levels can be used in place of echocardiography." It is unfortunate that not all practicing pediatricians or neonatologists have access to 24-hour/7-day-a-week echocardiography services. Serum BNP level is a useful adjuvant diagnostic tool that can be used in conjunction with a thorough physical examination, blood gas analysis, and other information to help physicians determine the proper treatment for the patient. Again, we emphasize that "[p]hysicians should have increased suspicion of PPHN, or other states of ventricular stress, when caring for a term or near-term newborn with RD [respiratory distress] and an elevated BNP level."

We feel that any infant with respiratory distress and an elevated BNP level should receive a thorough evaluation, likely including echocardiography, to determine the proper course of treatment. We are glad that preliminary data from Baptista et al support our hypothesis and that they agree with the conclusions we put forth in our article. We look forward to their final manuscript.

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doi:10.1542/peds.2005-0040

Detection of the *Bartonella henselae* Gene Sequence in Lymph Nodes of Children With Kikuchi's Disease

To the Editor.—

Kikuchi's disease (KD), also known as Kikuchi-Fujimoto disease or histiocytic necrotizing lymphadenitis, was first described in 1972 independently by Kikuchi[1] and Fujimoto et al.[2] Viral infection has been suggested as a possible etiology because of clinical manifestations such as fever, lymphadenopathy with a self-limited course, and occasional peripheral lymphocytosis. However, most studies failed to detect suspicious viral agents including Epstein-Barr virus, human herpesvirus 8, human herpesvirus 6, and parvovirus B19 in KD.[3] Some bacterial agents including *Yersinia enterocolitica*, *Toxoplasma*, and *Brucella* were occasionally identified by serologic methods.[3] Although KD is rare in children, it should be considered in the differential diagnosis of infections, collagen diseases, or malignancy. Cat-scratch disease (CSD) is a worldwide zoonosis caused by *Bartonella henselae* or *Bartonella clarridgeiae* and is characterized by a self-limiting regional lymphadenopathy associated with a cat scratch or bite. However, no history of animal contact can be elicited in a small percentage of patients with CSD. Atypical CSD is reported in up to 25% of cases. CSD is usually suspected clinically and confirmed by the detection of *Bartonella* DNA in lymph nodes by polymerase chain reaction (PCR). We investigated the presence of *B henselae* DNA in children diagnosed with KD. Twenty children who were diagnosed with KD at Sanggye-Paik Hospital, Inje University College of Medicine, from January 1998 to December 2004 were included in the study. DNA was extracted from lymph node specimens on slides and paraffin-embedded tissues. *B henselae* strain Houston was used for a positive control, and 2 different primers were used in this study. The PCR assay was performed by using the citrate synthetase (*glt*A) gene, an outer pair of primers consisting of TN-1 and TN-2, and an inner primer as described.[4] Seminested PCR protocols for amplification of the *pap*31 gene of *B henselae* with primers *PAP*n1, *PAP*n2, and *PAP*ns2 were as described.[5] The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. PCR products were sequenced in both directions, and sequence analysis was performed. The mean age of the 20 children with KD was 13 years (range: 5–17 years). Eleven patients were female. The lymph node lesions were located in the cervical area in 80% of the children, the submandibular area in 10%, and the axillary area in 5%. *B henselae* DNA was detected in 4 patients (25%). With the *glt*A primer, a positive PCR result was obtained from 4 patients (25%), whereas the *pap*31 primer amplified *B henselae* DNA in only 1 patient (5%). The amplification products were confirmed by sequence analysis. None of the negative controls were positive. We had performed seminested PCR assays by using different primers targeting the citrate synthetase (*glt*A) gene and *pap*31 gene of *B henselae* in lymph nodes of children with KD. A diagnosis of CSD by PCR from fine-needle aspiration and primary lesion specimens was reported to be minimally invasive and highly accurate.[6] Typical pathologic findings are characteristic of, but not specific for, CSD. The results of our study suggest that *B henselae* may be an etiologic agent in some children diagnosed with KD. Additional studies are needed to clarify the association of *B henselae* with KD.

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CHAPTER VI

PROGNOSTIC VALUE OF NT-PROBNP

IN HUMAN NEWBORNS WITH CONGENITAL DIAPHRAGMATIC HERNIA

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N-Terminal-pro-B Type Natriuretic Peptide as a Useful Tool to Evaluate Pulmonary Hypertension and Cardiac Function in CDH Infants

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Key Words
N-terminal-pro-B type natriuretic peptide · Congenital diaphragmatic hernia · Pulmonary hypertension · Cardiac function · Diastole

Abstract
Objective: In congenital diaphragmatic hernia (CDH) the severity of pulmonary hypertension (PH) is considered, by several authors, determinant of clinical outcome. Plasmatic N-terminal-pro-B type natriuretic peptide (NT-proBNP) might be useful in diagnosis and management of PH in newborns, although its interest in CDH infants remains to be defined. Early NT-proBNP levels were assessed in CDH infants and correlated with cardiovascular echocardiographic parameters. ***Patients and Methods:*** 28 newborns, CDH and age-matched controls were enrolled in a prospective study. Clinical condition, NT-proBNP plasmatic levels, echo parameters of PH and biventricular function were assessed at 24 h after delivery as well as survival outcome. ***Results:*** Estimated mean pulmonary pressure and NT-proBNP were significantly higher in CDH than control infants. NT-proBNP significantly correlated with estimated pulmonary artery pressure, right ventricular Tei index, and tricuspid E/A ratio. Additionally, we found that CDH infants with NT-proBNP >11,500 pg/ml experienced a worse prognosis. ***Conclusions:*** We demonstrated that PH is associated with NT-proBNP elevation and diastolic impairment in CDH infants. Early elevations in NT-proBNP levels seem to alert for a subset of CDH infants with worse prognosis.

Introduction

Despite many advances in the management of congenital diaphragmatic hernia (CDH), the outcome of affected infants remains unpredictable and varies from year to year even in experienced teams with predetermined treatment protocols. Its morbidity and mortality is largely dependent of pulmonary hypertension (PH) and hypoplasia [1]. Pulmonary hypoplasia installs during prenatal development [2–4] and limited possibilities exist to attenuate it [5, 6]. PH is likely secondary to pulmonary

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hypoplasia and associated underdeveloped vascular bed, which for some authors is a major determinant of postnatal clinical outcome [7]. For this reason, evaluation of PH severity is considered important for management of CDH infants in several centers [1] and its assessment is crucial to decide upon pulmonary vasodilator therapy as well as to monitor its effects [8, 9]. Furthermore, for these authors the decision for surgical repair should be based on evidence of PH stabilization [10]. Assessment of PH is mainly based on clinical and echocardiographic estimation. However, echocardiographic evaluation is not always available and it is somewhat observer-dependent and technically demanding in CDH infants due to the presence of abdominal organs in thoracic cavity. An easy and reliable method to assess PH remains to be defined. On the other hand, PH could induce cardiac dysfunction due to right ventricular (RV) overload, although this aspect has not been clearly elucidated in CDH infants [11].

B-type natriuretic peptide (BNP) is a hormone of predominantly ventricular origin produced and released in response to increased ventricular wall stress [12, 13]. N-terminal-pro-BNP (NT-proBNP), the amino-terminal portion of the preprohormone, is secreted into the peripheral blood in equimolar portions to BNP, but it has a longer half-life and is easier to measure [13]. In recent years, NT-proBNP has emerged as a very sensitive biochemical marker for ventricular dysfunction in adult heart failure, the plasmatic level of which could be used as a guide for the response to therapy and to predict prognosis [14]. However, in children the knowledge about the significance of plasma levels of NT-proBNP is still limited. In healthy children, studies have shown that NT-proBNP levels are elevated soon after birth reaching its peak at 24 h of life decreasing thereafter up to 4 months and remaining unchanged until the age of 15 [15–19]. NT-proBNP levels are elevated in children with congenital heart disease or cardiomyopathy [20–22]. In infants, it was also demonstrated that NT-proBNP is elevated in symptomatic patent ductus arteriosus in preterms [23, 24] and PH [25]. Additionally, in newborns submitted to cardiac catheterization due to critical pulmonary stenosis or atresia, NT-proBNP correlates with hemodynamic measured RV pressure [26]. However, assessment of plasmatic NT-proBNP and its clinical usefulness in newborns with PH due to CDH remains to be defined.

The aim of this study was to compare echocardiographic parameters of cardiac function and plasmatic NT-proBNP levels, at 24 h after birth, between CDH and healthy infants.

Materials and Methods

Study Subjects

From January 2004 to October 2006, we enrolled in this study term or near-term newborns (≥36 weeks' gestation) admitted to our hospital's neonatal intensive care unit (NICU) or normal newborn nursery. Two groups were defined: (1) a control group that included healthy infants, and (2) a CDH group that included consecutive newborns with left-sided CDH (Bochdalek hernia) without heart defect. Sample size was calculated in order to detect a difference of at least 2 units between groups' means, assuming a standard deviation of 1 unit, with a 95% confidence level (5% α level) and at least 85% power, in an independent samples Student's t test. Thus, we estimated having to recruit at least 20 newborns (10 CDH patients and 10 controls).

Healthy infants were identified from patients that needed blood sampling, at the second day of life, for clinical reasons not related to this study, specifically by suspected sepsis or physiologic jaundice. In these infants, echocardiography was performed immediately before blood was drawn for functional evaluation and exclusion of congenital heart disease. Physiologic jaundice is defined as a benign condition with bilirubin levels <15 mg/day. Infants with confirmed sepsis or newborns with transient tachypnea as well as congenital heart disease, PH or hemodynamic significant ductus arteriosus were excluded from this study.

In CDH newborns, echocardiography was done immediately before blood was drawn that is performed routinely at 24 h of life. Clinical management of CDH infants in our NICU include delayed surgical repair after extensive preoperative stabilization with 'gentle ventilation' and inhaled nitric oxide treatment, if necessary. We could not manage patients with extracorporeal membrane oxygenation (ECMO) since it is not available in our center.

NT-proBNP was measured in the blood drawn in these two groups.

Demographic data from both study groups included postmenstrual age at delivery, gender, birth weight as well as 1- and 5-min Apgar scores. At the moment of blood sampling, data gathered included systolic, mean and diastolic blood pressure, requirement of inotropic support, pulmonary vasodilator therapy as well as of mechanical ventilation, current ventilator settings and blood gases. In CDH infants the oxygenation index and the ventilatory index were calculated. Simultaneously, echocardiographic parameters and plasmatic NT-proBNP levels were recorded. Additionally, we recorded the day of surgery as well as survival taking into account that survivor newborn was defined as alive at discharge from NICU.

The study was approved by the Institutional Review Board of our hospital and informed consent was obtained from the parents of all participants. No infant, neither case nor control, received additional blood collection, other than routine blood sampling, as a consequence of this study, as NT-proBNP usually could be measured in the routine amount of blood sample. Data collected from this study were not used to influence medical decision-making. Management of each infant was left to the criteria of the attending physician, according to the treatment protocol of our NICU. NT-proBNP plasmatic measurement was not achieved in 5 CDH newborns, due to insufficient sample.

Echocardiographic Assessment

The echocardiographic assessment was designed as part of the infants' routine care or additionally to that, at no cost to the patient. All examinations were performed with an echograph Aloka (Tokyo, Japan) using a 5-MHz probe, by the same investigator (M.J.B.). Once echocardiography always preceded blood sampling, the investigator interpreting echocardiography was blinded to NT-proBNP levels, although not blinded for control versus CDH groups.

In infants from control and CDH groups, echocardiography was performed to exclude congenital heart disease and to ascertain the following parameters: (i) RV and left ventricular (LV) systolic and end-diastolic dimensions (M-mode, parasternal ventricular short-axis view); (ii) mitral and tricuspid diastolic dimensions (2D, 4-chamber view); (iii) dimensions of the right and left pulmonary arteries (RPA and LPA); (iv) orientation of ventricular septum (existence of bulging to left ventricle); (v) ductus arteriosus and foramen oval patency and shunt direction; (vi) existence and quantification of tricuspid regurgitation as well as RV-right atrium (RA) gradient; (vii) RV outflow acceleration time (OAT); (viii) RV outflow ejection time; (ix) peak flow velocity of A and E wave at mitral and tricuspid valves; (x) time from cessation of tricuspid and mitral inflow to onset of tricuspid and mitral inflow, respectively, in the next cardiac cycle. These allowed the estimation of: (xi) mitral-to-tricuspid ratio; (xii) LPA-to-RPA ratio; (xiii) estimated right ventricular systolic pressure (RVSP) through the formula: RVSP = tricuspid regurgitation gradient + RA pressure, assuming a normal RA pressure of 4 mm Hg [27, 28]; (xiv) estimated mean pulmonary artery pressure (MPAP), through the formula MPAP = 90 – (0.62 × OAT) [29]; (xv) tricuspid and mitral E/A ratios; (xvi) the RV and LV Tei index, as described by Tei et al. [30], assessed as Tei index = (time from cessation of tricuspid or mitral inflow to onset of tricuspid or mitral inflow in the next cardiac cycle – RV or LV outflow ejection time)/RV or LV outflow ejection time; (xvii) RV OAT to RV outflow ejection time, a quantitative predictor of peak PA pressure in infants [31]. Measurements were obtained in 3 consecutive cardiac cycles and averaged to account for respiratory variation.

Taking into account the distinct characteristics of RV and LV, we evaluate ventricular function using different parameters: (i) global function, using the Tei index in RV and LV [32]; (ii) systolic function, with peak pulmonary flow velocity in RV [33] and ejection fraction in LV; (iii) diastolic function, tricuspid and mitral E/A ratio in RV and LV, respectively.

In CDH newborns the echocardiographic window is limited due to the intrathoracic position of abdominal organs and mechanical ventilation. Nevertheless, the echocardiographic protocol was completed in all CDH patients. For that purpose the subcostal window or right-sided parasternal window was sometimes used instead of the classical echo windows.

NT-proBNP Measurement

The plasmatic level of NT-proBNP was evaluated at 24 h of life in every control infant and in 13 of the 18 CDH patients. NT-proBNP levels were measured with a chemiluminescent immunoassay kit (Roche Diagnostics, Portugal) on an Elecsys 2010 analyzer. Venous blood samples were collected in EDTA-containing tubes.

Statistical Analysis

Descriptive statistics were presented as mean and standard deviation as appropriate. Plasma NT-proBNP measurements resembled a log-normal distribution, so natural logarithmic transformation was used to normalize the distribution when indicated. Comparisons between groups were performed using Fisher's exact test for categorical variables. For continuous variables comparisons, we used Student's t test if normality of the distributions could be assumed or non-parametric Mann-Whitney U test if normality could not be assumed. In order to test the normality of the distribution of the continuous variables the one-sample Kolmogorov-Smirnov goodness-of-fit test was applied. Pearson correlation coefficients were calculated to evaluate the relationship between NT-proBNP and estimated pulmonary pressure as well as the echocardiographic parameters of cardiac function. To describe the discriminative power of plasma NT-proBNP measurement concerning mortality outcome, sensitivity and specificity were calculated for various levels of this variable, a receiver operating characteristic (ROC) curve was drawn. Given the small sample size it was not possible to analyze the prognostic value of plasma NT-proBNP measurements using appropriate multivariate modeling methods. For hypothesis testing, a value of $p < 0.05$ was considered significant.

Results

In this study, we evaluated 28 infants, 10 in the control group and 18 in the CDH group. In the CDH group, NT-proBNP was evaluated only in 13 newborns, due to an insufficient amount of blood for the measurement. The demographic data of CDH infants are presented in table 1. Excluding Apgar scores in CDH infants, no significant differences were identified with regards to birth weight, gestational age and gender.

The clinical conditions of CDH newborns, regarding the requirement of inotropic support, pulmonary vasodilator therapy as well as day of surgery and outcome, are summarized in table 2. NT-proBNP levels were significantly higher in CDH than in the control group (fig. 1).

Evaluated echocardiographic parameters in control and CDH groups are presented in table 3. In newborns with CDH, estimated MPAPs were significantly higher when compared to the control group reflecting an increased afterload to the RV. All newborns from CDH groups had some echocardiographic evidence of PH, bulging of interventricular septum and right-to-left shunt at the level of ductus arteriosus and foramen ovale. Morphologically, RV systolic and diastolic dimensions were significantly higher in the CDH group than the control group, whereas LV systolic and diastolic dimensions were significantly lower in the CDH than the control group.

Table 1. Demographic data

	Groups studied		p value
	control group (n = 10)	CDH group (n = 18)	
Birth weight, g	3,236 ± 612	2,954 ± 451	0.175[1]
Gestational age, weeks	38.3 ± 1.3	37.7 ± 1.7	0.430[1]
Male gender	9 (90)	12 (67)	0.364[2]
1-min Apgar, median	9	7	0.003[3]
5-min Apgar, median	10	8	<0.001[3]
Hours of measurement	26 ± 1.2	23 ± 0.9	0.463[1]

CDH = Congenital diaphragmatic hernia. For continuous variables, values are expressed as numbers of infants (%) or means ± SD.

[1] t test for independent samples. [2] Fisher's exact test. [3] Mann-Whitney non-parametric test.

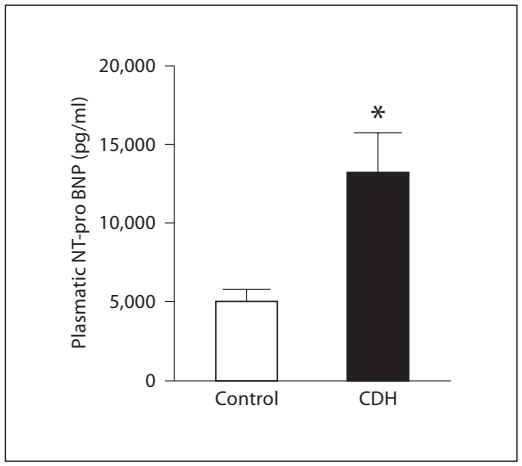

Fig. 1. Plasmatic NT-proBNP level is significantly increased in newborns with CDH compared to control (* $p < 0.05$ vs. control).

Table 2. Clinical data of CDH newborns

Infant	Inotropic support	Vasodilator therapy	Mechanical ventilation	Day of surgery	Day of discharge
1	Yes (dopamine and dobutamine)	Yes (iNO)	SIMV (for 24 days)	D14	D24 (death)
2	No	No	SIMV (for 10 days)	D7	D14 (alive)
3	Yes (dopamine and dobutamine)	No	SIMV (for 2 days)	No	D2 (death)
4	Yes (dopamine and dobutamine)	No	SIMV (for 2 days)	No	D2 (death)
5	No	No	SIMV (for 6 days)	D4	D13 (alive)
6	Yes (dopamine and dobutamine)	No	SIMV (for 12 days)	D4	D18 (alive)
7	No	No	SIMV (for 6 days)	D4	D11 (alive)
8	Yes (dopamine)	No	SIMV (for 7 days)	D4	D24 (alive)
9	Yes (dopamine and dobutamine)	No	SIMV (for 3 days)	D3	D3 (death)
10	No	No	SIMV (for 14 days)	D5	D21 (alive)
11	Yes (dopamine and dobutamine)	Yes (iNO)	HFVO (for 89 days)	D42	D89 (death)
12	Yes (dopamine and dobutamine)	Yes (iNO)	HFVO (for 20 days)	D9	D38 (alive)
13	Yes (dopamine)	No	SIMV (for 7 days)	D3	D17 (alive)
14	Yes (dopamine)	Yes (iNO)	SIMV (for 6 days)	No	D6 (death)
15	Yes (dobutamine)	Yes (iNO)	SIMV (for 35 days)	D14	D46 (alive)
16	Yes (dopamine)	Yes (iNO)	SIMV (for 8 days)	D8	D21 (death)
17	Yes (dopamine)	Yes (iNO)	SIMV (for 11 days)	No	D11 (death)
18	Yes (dopamine)	Yes (iNO)	SIMV (for 13 days)	D4	D13 (death)

iNO = Inhaled nitric oxide; SIMV = synchronous intermittent mandatory ventilation; HFOV = high-frequency oscillatory ventilation.

Additionally, mitral-to-tricuspid and LV-to-RV diastolic M-mode ratios were significantly lower in the CDH than the control group.

Evaluation of global function parameters revealed that the RV Tei index was significantly higher in the CDH than the control group. Regarding systolic function, in comparison to the control group, CDH infants had a slightly depressed RV function as evidenced by a significant decrease in peak pulmonary velocity, whilst the LV systolic function was enhanced as demonstrated by a higher LV ejection fraction. Both RV and LV diastolic parameters (E/A auriculo-ventricular ratios) were signifi-

Table 3. Data of echocardiography in control and CDH infants at 24 h after birth

	Control group	CDH group	p[a]
Pulmonary pressure			
Pulmonary systolic pressure, mm Hg[b]	–	56.0 ± 18.6	
Pulmonary mean pressure, mm Hg[b]	41.6 ± 14.9	64.0 ± 14.9	0.001
Heart dimensions			
Right ventricle			
Tricuspid diameter, mm	9.45 ± 1.00	10.45 ± 2.00	0.055
RV systole M-mode, mm	5.75 ± 2.70	9.40 ± 4.70	0.017
RV diastole M-mode, mm	7.00 ± 2.15	11.30 ± 3.60	0.009
Left ventricle			
Mitral diameter, mm	9.90 ± 1.80	8.15 ± 1.80	0.019
LV systole M-mode, mm	10.40 ± 1.10	7.90 ± 3.90	0.023
LV diastole M-mode, mm	16.00 ± 1.40	14.30 ± 3.50	0.025
Ratios			
Mitral-to-tricuspid	1.03 ± 0.15	0.79 ± 0.15	<0.001
RPA-to-LPA	1.15 ± 0.30	1.10 ± 0.20	0.071
LV-to-RV diastole M-mode	2.35 ± 0.65	1.10 ± 0.40	0.002
Heart function			
Global function			
RV Tei index	0.21 ± 0.05	0.28 ± 0.09	0.002
LV Tei index	0.22 ± 0.17	0.23 ± 0.10	0.674
Systolic function			
Peak pulmonary velocity, m/s	0.80 ± 0.18	0.59 ± 0.12	0.003
LV ejection fraction, %	67 ± 9	80 ± 17	0.02
Diastolic function			
Tricuspid E/A ratio	0.96 ± 0.11	0.80 ± 0.13	<0.001
Mitral E/A ratio	1.00 ± 0.44	0.81 ± 0.13	0.019

Results are presented as median ± interquartile range.

CDH = Congenital diaphragmatic hernia; RPA = right pulmonary artery; LPA = left pulmonary artery; LV = left ventricle; RV = right ventricle.

[a] Mann-Whitney non-parametric test. [b] Both mean and systolic pulmonary pressure were estimated.

cantly lower in the CDH group than the control group, suggesting significant RV and LV diastolic impairment in the CDH group. We found significant correlations between NT-proBNP and estimated pulmonary mean pressure ($r = 0.45$; $p = 0.03$), RV Tei index ($r = -0.50$; $p = 0.02$) and tricuspid E/A ratio ($r = -0.46$; $p = 0.03$).

Comparison between Survivors and Non-Survivors

Nine infants of the CDH group died. A comparison between survivors and non-survivors is presented in table 4. NT-proBNP plasmatic level, at the end of the first day of life, was significantly higher in non-survivor than in survivor newborns. There are no differences between both groups concerning birth weight, gestational age, 1- and 5-min Apgar or mean arterial blood pressure. Regarding pulmonary indices, we detected significant differences between survivor and non-survivor infants. On echo parameters, we identified that estimated mean pulmonary pressures were significantly higher in non-survivor than in survivor CDH infants, whereas pulmonary acceleration to ejection time and tricuspid E/A ratios were significant lower in non-survivor than in survivor CDH infants.

The NT-proBNP level of 11,500 pg/ml was the value with highest specificity and sensitivity to separate survivor and non-survivor CDH infants in ROC curves, in CDH infants. According to the ROC curve, the cutoff NT-proBNP level of 11,500 pg/ml has 100% sensitivity and 67% specificity. Infants with a plasmatic NT-proBNP at 24 h of life >11,500 pg/ml had a significantly lower survival rate than those with plasmatic NT-proBNP at 24 h of life <11,500 pg/ml.

Table 4. Parameters studied at 24 h of life in newborns with CDH according to survival

	Survivors (n = 9)	Non-survivors (n = 9)	p
NT-proBNP, mg/ml	6,230 ± 4,743	22,653 ± 5,653	0.009[2]
log[NT-proBNP] *	8.73 ± 0.87	10.14 ± 0.30	0.003[1]
Demographic data			
Birth weight, g *	3,098 ± 415	2,882 ± 620	0.238[2]
Gestational age, weeks *	38 ± 1.4	37 ± 2.5	0.686[1]
Male gender, n (%)	6 (67)	6 (67)	0.999[3]
1-min Apgar	8 ± 1	7 ± 3	0.21[2]
5-min Apgar	9 ± 1	8 ± 3	0.178[2]
Mean arterial blood pressure, mm Hg *	40 ± 8	41 ± 10	0.449[1]
Pulmonary indices			
PaO_2	80 ± 20	42 ± 16	0.003[2]
$PaCO_2$	46 ± 15	58 ± 16	0.063[2]
FiO_2	0.3 ± 0.6	1.0 ± 0.0	0.002[2]
MAP	6.9 ± 1.9	12.8 ± 1.5	0.003[2]
Oxygenation index	2.2 ± 5.0	28.8 ± 24.26	0.003[2]
Ventilatory index	243 ± 141	879 ± 139	0.003[2]
Echo indices			
Mitral-to-tricuspid ratio	0.82 ± 0.20	0.77 ± 0.07	0.596[2]
LV-to-RV diastole M-mode ratio	1.2 ± 1.0	1.1 ± 0.2	0.400[2]
Pulmonary systolic pressure, mm Hg	42 ± 19	58 ± 19	0.077[2]
Pulmonary mean pressure, mm Hg	53 ± 11	68 ± 0.6	0.009[2]
RV Tei index	0.27 ± 0.05	0.31 ± 0.11	0.411[2]
LV Tei index	0.22 ± 0.09	0.23 ± 0.07	0.999[2]
Peak pulmonary velocity, m/s	0.58 ± 0.17	0.62 ± 011	0.700[2]
Pulmonary acceleration/ejection time	0.33 ± 0.1	0.22 ± 0.03	0.002[2]
Tricuspid E/A ratio	0.85 ± 0.11	0.76 ± 0.29	0.020[2]
Mitral E/A ratio	0.82 ± 0.15	0.79 ± 0.23	0.191[2]

Results are presented as median ± interquartile range unless otherwise indicated. * mean ± standard deviation.

[1] t test. [2] Mann-Whitney non-parametric test. [3] Fisher's exact test.

PaO_2 = Oxygen arterial partial pressure; $PaCO_2$ = carbon dioxide arterial partial pressure; MAP = mean airways pressure; LV = left ventricle; RV = right ventricle.

Discussion

In our study, we demonstrated that PH is associated with diastolic impairment and higher NT-proBNP levels measured at 24 h of life elevation in CDH infants. Early elevations in NT-proBNP levels seem to alert for a subset of CDH infants with worse prognosis.

In recent years, PH has emerged for many authors as a key determinant of outcome in CDH infants [1]. Consequently, PH progressively becomes one of the therapeutic targets in managing these newborns. In fact, in several therapeutic approaches (delayed surgery, high-frequency ventilation, nitric oxide inhalation, ECMO and other pulmonary vasodilators), one of the aims is controlling PH, whereas the decision for surgical repair is based on evidence of PH stabilization [9]. Since accurate evaluation of pulmonary artery pressure with a Swan-Ganz catheter, in newborns, is not achievable, echocardiography is used to assess PH. Nevertheless, echocardiography is not available 24 h a day in all centers dealing with CDH infants and reliable parameters are not easy to quantify, most of them being observer-dependent. In this setting, non-invasive and examiner-independent parameters could be particularly useful to manage CDH infants with PH, but should be available in a short time frame [34].

In this context, we investigated the potential significance of plasmatic NT-proBNP levels in CDH infants. The elevated levels of NT-proBNP that we found in CDH infants should be secondary to PH. Previous studies have demonstrated both in adults [35] and infants [24] that plasmatic NT-proBNP levels are elevated in patients with

PH. Although already demonstrated in adults, the correlation between pulmonary artery pressure and NT-proBNP has not previously been established in infants. In the present study we demonstrated that both in controls and CDH infants the plasmatic NT-proBNP level correlates with estimated mean pulmonary pressure. In CDH infants, invasive measurement of pulmonary pressure and hemodynamic parameters of cardiac function is not feasible. Currently, magnetic resonance imaging is employed in children and adults to evaluate RV, but this is also not reasonable in critical CDH infants.

We decided to measure the NT-proBNP at 24 h of life basically for two reasons: (1) it has been previously demonstrated that NT-proBNP reaches its peak by the end of the first day of life in healthy infants [15], and (2) it is well known that a number of CDH infants do well during the first day of life ('honeymoon period'), but after this period the clinical status of the most severely affected subset of CDH infants deteriorates. For these reasons, the evaluation of NT-proBNP at the end of the first 24 h of life appears appealing, since it could alert for those babies that will require more sophisticated methods to support their life such as ECMO. In fact, we hypothesize that the measurement of NT-proBNP could separate two groups of CDH infants with different prognoses. In our study, plasmatic NT-proBNP >11,500 pg/ml selected those CDH infants that experienced a worse prognosis.

In our study, we performed an exhaustive cardiac function assessment by echocardiography which revealed significant adaptation in biventricular function, both in systole and diastole. The systolic RV function is impaired in CDH infants, probably in relation with higher RV afterload. In CDH infants we found an increase in LV ejection fraction, which could be related with relative underfilling of the LV as well as with end-systolic septal bulging [36]. In fact, it was previously demonstrated that the end-systolic leftward ventricular septal shift in situations with RV pressure overload results in isolated augmentation of systolic shortening in the septal-to-free wall dimensions [36]. Whereas the potential mechanisms for LV filling abnormalities have been pointed out above, impairment of RV diastolic function might be directly related with increased RV afterload. In fact, afterload is a major determinant of diastolic function. Severe afterload elevations slow down the relaxation rate and elevate diastolic filling pressures due to an upward shift of the end-diastolic pressure-volume relation [37, 38]. Additionally, hypoxia and acidosis, reflected as lower Apgar indices observed in CDH newborns, also impair myocardial relaxation and might therefore further contribute to the impairment of both RV and LV diastolic function. The echocardiographic pattern of impaired relaxation (E/A <1) observed in both ventricles of CDH patients in the present study further reinforce our hypothesis. Global RV function evaluated by the Tei index is depressed in CDH infants. This finding is in line with the recent studies of Grignola et al. [39] who demonstrated that the RV Tei index is a sensitive marker of RV dysfunction in the settings of acute PH.

In the present study we documented a significant correlation between NT-proBNP and estimated mean pulmonary pressure as well as some echocardiographic indices of ventricular function (as RV Tei index and tricuspid E/A ratio). The increase of plasmatic NT-proBNP probably reflects ventricular adaptation to pressure overload secondary to PH and could be defined as a non-specific parameter of RV overload. Plasmatic NT-proBNP probably fluctuates according to the clinical condition of CDH newborns, and therapeutic management that modifies ventricular overload could change the level of this biochemical marker. Nevertheless, it will always reflect the actual hemodynamic status of the infants and their response to the treatment.

As previously referred to by several authors, in our study we documented that non-survivor CDH newborns required more aggressive ventilation with impaired blood gas exchange. In fact, some parameters derived from oxygenation status had been suggested as a potential marker of prognosis [40]. The comparison of survivor and non-survivor CDH infants demonstrated that NT-proBNP and estimated pulmonary mean pressure were significantly different in both groups, probably reflecting the increasing severity of the disease. Nevertheless, with the exception of pulmonary acceleration to ejection time and E/A tricuspid ratios, no other parameters of heart function were significantly different in both groups. We believe that this is due to a failure of echocardiography to accurately evaluate heart function and effectively differentiate degrees of severity in impairment of heart dysfunction, instead of lack of increased adjustments in cardiac function related to PH.

In conclusion, we demonstrated that PH in CDH infants induces cardiac function adaptations, which could be evaluated not only by echocardiography but also by plasmatic NT-proBNP. Although this study was carried out in a small number of patients, this biochemical marker could be an accurate indicator of the severity of the clinical condition and PH in CDH newborns. Thus, it seems reasonable to suggest that CDH infants with early

elevations in NT-proBNP should alert for a subset of CDH infants with worse prognosis that should be considered for further study, pulmonary vasodilator therapy or transfer to an ECMO center.

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CHAPTER VII

GENERAL DISCUSSION AND BIBLIOGRAPHY

In last decades the human knowledge in science and medicine has evolved exceedingly. In clinical practice, health indicators and gold standards improved and consequently, the expectation from health professionals and patients increased. Nevertheless, although those advances contributed to a general low perinatal morbidity and mortality, we still have huge challenges like CDH infants. In fact, time to time, despite the tremendous effort from a multidisciplinary team, we lose a CDH infant. Regardless the frustration behind our inability to recover these patients, there is a need for better understanding the mechanisms of the disease in an attempt to progress in therapeutics and improve the prognosis of CDH. This justifies the enormous efforts as well as the experimental and clinical investigation in this particular field.

The clinical presentation of CDH infants is very heterogeneous and the outcome of newborns is sometimes quite surprising. At one extreme of the spectrum of the disease there are newborns minimally affected with excellent prognosis after surgical correction of the diaphragmatic defect. At the opposite, we find severe CDH cases with multiples associated anomalies particularly chromossomic and/or cardiac malformations, which determine a poor prognosis that could even result in fetal demise. In the middle range, we found CDH *classic* infants without congenital heart malformation, but with severe pulmonary hypoplasia and PH that require intensive and sophisticated treatment in addition to the straightforward surgical diaphragmatic correction. These newborns represent the greatest challenge for those caring CDH infants, because if they survive to the critical neonatal period, lung will growth progressively and resolution of increased vascular resistance will occur with good long term prognosis. This particular subset of CDH infants were the main subject of study of this thesis.

Ten years ago when our group started CDH research, the real significance of heart involvement was emerging [Fauza *et al.*, 1994; Thébaud *et al.*, 1997]. The possible association with congenital heart diseases and its ominous prognosis was already well known and pediatric cardiologist routinellly screen heart malformations by echocardiography. At that time, many authors revised their institutional protocols in order to pregnancy interruption for those fetuses with prenatal diagnosis of CDH and cardiac malformations. Subsequent studies analyzed the cardiac morphological defects associated with CDH and noticed that most of them were conotruncal defects, such as TOF, vascular rings, and anomalies of the aortic arch [Graziano *et al.*, 2005]. Interestingly, these defects are greatly related with an abnormal neural crest migration which is signaled by the retinoids like vitamin A [Dickman et al., 1997; Niederreither *et al.*, 2001]. After achievement of this step, clinicians stressed the role of congenital heart diseases in CDH and no additional information to morphological normality was expected from fetal or postnatal echocardiography.

The close association between conotruncal cardiac malformations, lung hypoplasia and diaphragmatic hernia gave support to the hypothesis that retinoid signaling could play a major role in CDH syndrome. The retinoid hypothesis was not totally novel. In fact, Wilson *et al.* in 1953 described that vitamin A deficiency in pregnant rat females leads to several malformations in rat pups including diaphragmatic defects [Wilson *et al.*, 1953]. More recently, several authors observed both in experimental and clinical studies several evidences of the association between vitamin A and CDH as well as lung hypoplasia [Mendelsohn *et al.*, 1994; Thébaud *et al.*, 1999]. Thus, the relevance of retinoid signaling in lung, heart and diaphragmatic development was extensively investigated in an attempt to find the cause of CDH and possible therapeutic targets [Thébaud *et al.*, 1999; Major *et al.*, 1998; Greer *et al.*, 2003; Montedonico *et al.*, 2006]. These studies have been encouraged as several new gene mutations were being described involving genes for transcription of vitamin A binding proteins in

human CDH infants [Holder *et al.*, 2007]. Interestingly, in the nitrofen-induced CDH rat model, that shares several similarities with the human disease, it was also established that nitrofen interferes with normal retinoid signaling [Noble *et al.*, 2007].

It was demonstrated that supplementation with vitamin A could diminished the incidence of diaphragmatic defect in rat pups exposed to nitrofen, but the effect on heart and lung development was not entirely evaluated [Thébaud et al., 1999; Babiuk *et al.*, 2004]. Aiming to contribute for this subject, we investigated what would be the best time for vitamin A therapy in CDH fetuses in the nitrofen-induced CDH rat model. In an experimental study, we confirmed that administration of vitamin A early in fetal development partially prevented the diaphragmatic defect and improved lung growth. This effect was only partial, very early in gestation, suggesting that vitamin A only interfere with precocious molecular mechanisms involved in lung hypoplasia [Baptista *et al.*, 2005]. In our opinion, these results had clinical implications since it precludes the utilization of vitamin A as pharmacological therapeutics to improve lung growth. The fetal diagnosis of CDH in humans occurs only after 16 weeks gestation, when the main determinants of lung hypoplasia are mechanical. In addition, the utilization of vitamin A in humans at so early gestation dates involves an unacceptable risk of other malformations, ethically intolerable. Although this work does not support prenatal therapy of CDH fetuses with retinoids, it does not refute the hypothesis of fetal therapy based in growth factors. For instances, recent studies from our group found potential benefits from prenatal growth factor therapy using ghrelin [Henriques-Coelho *et al.*, 2004; Santos *et al.* 2006] and even cytokines [Nogueira-Silva *et al.*, 2006].

Regarding the heart development in the experimental vitamin A protocol, we didn't found any effect of vitamin A in heart growth (non published data), despite its effect in lung growth. With these results we suggested that the mechanisms involved in the

lung hypoplasia did not affect the heart growth and reinforced our results that heart doesn't present any hypoplasia at the end of gestation. Moreover, this study allowed us to suggest that fetal heart underdevelopment in contrast to lung hypoplasia can not be explained by the *two-hit hypothesis*.

In previous works from our group, we tried to identify morphological evidence of left heart hypoplasia in the experimental rat model of CDH, but unexpectedly to us in an exhaustive study performed during gestation, in nitrofen rat model, excluding hearts with malformations, we only found heart hypoplasia early in gestation, with total normalization of heart development until term [Correia-Pinto *et al.*, 2000; 2003]. The *dual hit hypothesis* defined that in CDH two insults occurs during lung development, the first one occurs early in gestation and is probably related with the molecular mechanisms in the origin of CDH, and the second occurs later in gestation and is related to the mechanical effect of abdominal organs compressing the lung [Keijzer *et al.*, 2000]. On the contrary, in the nitrofen rat model the heart seems to be affect early in gestation, recovers during gestation, despite the mechanical effects that limit the parallel lung growth [Correia-Pinto *et al.*, 2003]. Probably, the higher pressure in cardiac chambers precludes its compression. Curiously, more recent studies in humans, from several groups corroborate our findings, excluding heart hypoplasia and the clinical value of those echocardiographic parameters [Lin *et al.*, 2007].

The absence of heart hypoplasia doesn't exclude the existence of some molecular heart immaturity that could interfere with cardiac perinatal adaptation, causing hemodynamic instability in CDH infants. A few studies from one research team suggested cardiac molecular immaturity in CDH. These studies were performed in the experimental model of CDH induced by the nitrofen and demonstrated that the myocardium expression of several growth factors (TGFbeta, IGF-1, epidermal growth

factor, basic fibroblast growth factor and platelet-derived growth factor) were decreased in CDH fetuses compared to control [Guarino *et al.*, 2000, 2001; Teramoto *et al.*, 2001, 2005]. In the nitrofen rat model of CDH induced by the nitrofen, pregnant female's rats receive by gavage the teratogenic nitrofen, in a well defined time point of gestation (9½ dpc), causing the CDH syndrome in half of the exposed fetuses [Kluth *et al.*, 1990; Tenbrinck *et al.*, 1990]. Studies in this models requires the comparison not only between CDH and control pups but also between CDH and pups exposed to nitrofen without diaphragmatic defect (the *nitrofen group*). Nevertheless, in the published studies suggesting heart immaturity in CDH, the CDH pups were only compared to control groups with evident under-expression of defined growth factors. The absence of comparison of CDH group with nitrofen group could represent a significant limitation of these studies, since the observed underdevelopment could be related only with the effect of nitrofen, and not with real immaturity associated with CDH. On the other hand, those studies had been carried out only in fetuses at term. For those reasons we decided to further investigate the presence of molecular heart immaturity in CDH.

Heart development evolves with alterations in cardiomyocyte-specific gene expression. These adaptations occur at different levels either at cell-to-cell communication as well as at intracellular calcium kinetics and contractile proteins [Chen *et al.*, 2000; Kaba *et al.*, 2001; Reiser *et al.*, 2001]. We performed a study evaluating, in the nitrofen-induced CDH rat model, gene expression of cardiac molecular parameters that are developmentally regulated and reflect each of those levels of adaptations: i) connexin 43 mRNA, to cell-to-cell communication; ii) SERCA2A mRNA, to intracellular calcium kinetics; iii) myosin heavy chain protein isoforms, to contractile proteins. We didn't found any molecular signs of myocardium immaturity in this experimental model of CDH, since the molecular parameters were

similar in all studied groups (control, nitrofen and CDH groups) [Baptista *et al.*, 2006]. With these results, we exclude the hypothesis that heart hypoplasia or immature could be the *missing link* in CDH responsible for greater mortality. Nevertheless, in our clinical practice we progressively recognize that CDH newborns present acute heart failure. Since heart hypoplasia and underdevelopment were excluded, we realize that this might be caused mainly by cardiac overload secondary to severe PH.

Pulmonary vascular abnormalities in CDH are described by several authors [Geggel *et al.*, 1985; Beals *et al.*, 1992; Roubliova *et al.*, 2004; Taira *et al.*, 1998]. In CDH human fetuses, the pulmonary vascular tree demonstrates a developmental arrest of arterial branching at 12–14 weeks' gestation. The diameter of the vessels is decreased in relation to lung volume. This results in a decreased cross-sectional area of the vascular bed, with increase in pulmonary vascular resistance. In some infants, intraacinar arteries have a muscular wall, whereas other infants have no distal muscularization. Muscular wall thickness and muscle mass are increased in arteries bilaterally, compared to normal controls, and this increase in muscle mass is inversely proportional to the degree of lung hypoplasia.

Vascular anomaly that occurs during fetal development in CDH causes severe PH in affected newborns which has been proposed as one of the main determinants of mortality [Dillon *et al.*, 2004]. Pulmonary hypertension, beside interfere with gas exchange, causes right-to-left shunt at *foramen ovale* and *ductus arteriosus*, inducing hypoxia and acidosis which in turn aggravate pulmonary vascular constriction [Correia-Pinto, 2003]. In these patients, cardiac performance could be hampered due to increased RV afterload and impaired myocardial tissue oxygenation, which could be responsible for additional morbidity. It is well known that PH from other etiologies, in children and adults, causes RV adaptation, with hypertrophy and dilatation, that

progress to heart failure. Additionally, several studies suggest that in PH the LV also suffers both systolic and diastolic function adaptation, due to bulging of ventricular septum and reduced RV output [Louie *et al.*, 1995]. Nevertheless, the significance of pulmonary vascular anomalies and PH in heart function during perinatal development in CDH was not considered until recently. Thus, when we started assessment of heart function in CDH, two key questions were in our mind: i) *do pulmonary vascular anomalies causes any repercussion in heart function during prenatal development?* ii) w*hat is the impact of PH in heart function after birth?*

There are very limited data regarding fetal pulmonary vascular function in lung hypoplasia. In one limited study it was demonstrated in two fetuses with CDH and autopsy-proven pulmonary hypoplasia, that pulsatility index in the branch PA was elevated in utero [Chaoui *et al.*, 1999]. A subsequent study, evaluating reactivity of the pulmonary vasculature in response to maternal hyperoxygenation, demonstrated a higher mortality in fetuses with a non-reactive test and lung hypoplasia [Broth *et al.*, 2002]. Despite those, the impact of pulmonary vascular hypoplasia and increased vascular resistance in heart function during prenatal heart development was largely unknown.

The evaluation of heart function in experimental animal models is complex and not easily assessed by ultrasonography. Assessment of several biochemical markers of ventricular overload in the experimental model of CDH might provide an acceptable alternative to echocardiography. Several biochemical and genetic markers have been suggested to evaluate ventricular load and function, both in animal models and humans, like BNP, components of renin-angiotensin system and ET-1 [Miyauchi *et al.*, 1993; Lorrel, 1995; Nagaya *et al.*, 2000; Aubert, 2005]. The genetic expression of those proteins in fetal heart from early stages of development was already demonstrated [Beinlich *at al.*, 1993; Cameron *et al.*, 1996; Brand *et al.*, 2003], but its

cardiac expression during late stages of pregnancy is far from being clear, even in normal fetuses. In the experimental nitrofen rat model of CDH we determined the cardiac expression of BNP, angiotensiogen and ET-1 mRNA during perinatal development in normal and nitrofen-exposed rats [Baptista *et al.*, in press].

In control rat fetuses, cardiac expression of these genes showed temporal changes, suggesting a closely regulated developmental expression. Interestingly, we found a ventricular *mirror-image* expression of angiotensinogen and BNP: when BNP mRNA increases, angiotensinogen mRNA decreases. The opposed effect of natriuretic peptides and angiotensin in myocardium as well as in vessels is well known. Generally, BNP have potent inhibitory effects on the renin–angiotensin–aldosterone system [Burger *et al.*, 2005] and, in heart failure, activation of the renin–angiotensin–aldosterone system is suppressed by BNP [Takahashi *et al.*, 2003]. These systems might form an important regulatory complex of fetal vascular physiology and development. Hypothetically upheaval in that delicate control network could interfere in the cardiopulmonary hemodynamic and lead to disease.

Regarding nitrofen-exposed fetuses, our results showed late fetal cardiovascular molecular changes that were probably related with nitrofen. In fact, during fetal life we didn´t found any difference in genic expression of BNP, angitensinogen or ET-1 between CDH and nitrofen groups, excluding the hypothesis of fetal heart adaptations linked to CDH. Remarkably, the pattern of opposed direction of expression of BNP and angiotensinogen, observed in control rat fetuses, is preserved in nitrofen exposed fetuses. On the contrary to fetal results, we found RV molecular adaptation to pressure overload in CDH pups after birth, with increased myocardial expression of all studied genes compared to prenatal expression and compared to both control and nitrofen groups. This response was specific for RV and in this model we did not demonstrate any LV molecular adaptation to PH immediately

after birth. Our experimental study demonstrated that PH cause neonatal heart molecular adaptation, but the presence of heart dysfunction was still unrevealed.

The relevance of identify heart failure in human CDH infants is of utmost importance. Besides better understanding of the disease mechanisms it could have some clinical impact in the handling of the affected patients. Additionally, if heart function is partially dependent of pulmonary pressure, the evaluation of heart function could predict the severity of PH. In the absence of effective treatment of lung hypoplasia, the post natal manipulation of pulmonary vascular resistance is the foremost objective in CDH management. In this regard, PAP assessment is crucial to decide upon pulmonary vasodilator therapy as well as to monitor its effects. This requirement implies that the quantification of PAP and the assessment of heart function should be done using exact parameters, generally using echocardiography. However, several aspects should be considered in relation with echocardiography, namely in PAP estimation and heart function evaluation in newborns.

In experienced hands, echocardiography is a safe and adequate method to evaluate heart anatomy and function. Nevertheless, it is somewhat observer-dependent and technically demanding in CDH infants, due to mechanical ventilation and the presence of abdominal organs in thoracic cavity. Considering PAP estimation, it is based on tricuspid regurgitation using Doppler echocardiography and it is essential an exact alignment of the Doppler sample to achieve the greater gradient between RV and atrium. In CDH patients, the distortion of the heart frequently difficult the correct sample alignment with gross errors in estimated PAP. Additionally, the presence of TR is not universal in all patients with PH, and usually doesn't exist in healthy individuals. For all this reasons, in our studies we evaluated both the estimated pulmonary systolic pressure and also the estimated mean pulmonary pressure, based on acceleration time of pulmonary flow. The evaluation of PH was

also complemented with indirect signs, like shunt direction at *foramen ovale* and *ductus arteriosus*, the bulging of interventricular septum, as well as RV dilatation. Regarding evaluation of heart function with conventional echocardiography it is very challenge the assessment of RV due to its geometrical characteristics. Additionally, several heart function parameters defined in adults are uncertain in newborns due to normal heart function adaptation that occurs after birth. In fact, transition to post-natal life is accomplished by vascular and pulmonary adaptations, with particular load conditions that could interfere in evaluated parameters. In our clinical study, to evaluate heart function we selected echocardiographic parameters previously defined [Kinsella *et al.*, 1992; Sugiura *et al.*, 2004]. Right ventricle was evaluated with pulmonary acceleration time-to-ejection time (for systolic function), E-to-A ratio on tricuspid valve (for diastolic function) and RV Tei index (for global function). Left ventricle was evaluated with LV ejection fraction (for systolic function), E-to-A ratio on mitral valve (for diastolic function) and LV Tei index (for global function) [Tei *et al.*, 1997]. Despite this echocardiographic assessment, due to all previously explained limitation, easy and reliable methods to assess PH and its impact in cardiac function, in addition to conventional echocardiography, are required.

Several recent studies have demonstrated the clinical relevance of biochemical markers to evaluate and determine the prognosis in patients with heart failure and PH [Nagaya *et al.*, 2000; Bettencourt, 2004; McLaughlin *et al.*, 2004]. Several molecules which can be measured in the blood or sometimes in other biological fluids are known to be elevated in PH [Voelkel *et al.*, 2000; Torbicki *et al.*, 2003; Aubert, 2005; Abdul-Salam *et al.*, 2006]. From several biochemical markers (uric acid, troponins), NT-proBNP is considered the most useful biochemical marker of ventricular overload in addition to echocardiography in adults. At the beginning of our studies, little information was available about NT-proBNP in infants. In a small group of newborns with critical pulmonary stenosis or atresia, submitted to cardiac

therapeutic catheterization we found a significant positive correlation between plasmatic NT-proBNP and RV systolic pressure [Baptista *et al.*, 2005]. In the meantime, several authors demonstrated the significance of NT-proBNP in ventricular overload in infants and children [Reynolds *et al.*, 2004; Nir et al., 2004].

In an extensive clinical study we evaluated during two years every CDH infants admitted to our center. We assessed 18 CDH infants, at 24 hours of age. This time point was decided because during the first day of life these babies usually present hemodynamic and respiratory stability, the *honeymoon period*, and only after that stage the significance of PH could be ascertain. In selected infants, heart function was evaluated with several echocardiographic parameters of ventricular systolic and diastolic function. In those patients, plasmatic NT-proBNP was measured at the same time point. The results were compared to an age-matched healthy group, needing blood collection for other reasons. The results from our clinical study revealed that CDH infants presented ecocardiographic and biochemical evidence of heart dysfunction in relation with severity of PH. The echocardiographic assessment revealed that heart adaptation occurs in systolic and diastolic function of both ventricles, but mainly in RV. Interestingly, we found significant differences between CDH and control infants in echocardiographic parameters but no difference was found between CDH survivors and non-survivors. Regarding biochemical markers we found a significant increased of NT-proBNP in CDH infants in comparison to control newborns, but this difference is enlarged when we compare CDH survivors and non-survivors. In fact, NT-proBNP significantly correlates with estimated pulmonary pressure but doesn't have correlation with measured echocardiographic parameters of heart function. We found an excellent correlation between NT-proBNP and echo parameters of heart function in CDH survivors and control groups, but this correlation is absolutely loosed in CDH non-survivors group [non published data]. Plasmatic NT-proBNP seems to be more effective than echo parameters of heart function to

evaluate the hemodynamic impact of PH. We believe that plasmatic NT-proBNP should be added to echo parameters to assess heart function in order to closely follow up PH and its repercussion in heart function.

Thus, in the sequence of our work we suggest one protocol the evaluate PH and heart function in CDH newborns, in a simple and feasible way. This protocol should be followed in a multidisciplinary approach in order to improve PH assessment and manipulation.

Clinical protocol to cardiac assessment in CDH

1. Fetal assessment
    a. 20 weeks gestation (or as soon as diagnosis of CDH)
        i. Screening of congenital heart malformation
            1. Cono-truncal anomalies
            2. Associated anomalies (e.g., minor VSD)
        ii. *Evaluation of heart hypoplasia (interest not yet established)*
        iii. Heart and vascular function (not established)
            1. Pulmonary pulsatility index
    b. Serial evaluation until term
2. Neonatal assessment
    a. First day of life
        i. <u>ECHO</u>
            1. Screening of congenital heart malformation
            2. Pulmonary pressure estimation
                a. Pulmonary artery systolic pressure (PASP)
                b. Pulmonary artery mean pressure (PAMP)
    b. Second day of life (around 24 hours)
        i. <u>Biochemical</u>
            1. NT-proBNP
            2. Uric acid (not established)
            3. Troponin T (not established)
        ii. <u>ECHO</u>
            1. Pulmonary artery pressure quantification
                a. Estimation of PASP and PAMP
                b. Shunt direction at *foramen ovale* and *ductus arteriosus*
                c. Interventricular septum direction
                d. RV dilation (tricuspid-to-mitral ratio)

2. Heart function
    a. Right ventricle
        i. RV acceleration time/ejection time
        ii. E/A wave ratio
        iii. Tei index
    b. Left ventricle
        i. Ejection fraction
        ii. E/A wave ratio
        iii. Tei index

c. Repeat BIOCHEMICAL and ECHO (same as day 2):
    i. Clinical deterioration/follow-up (dependent of clinical status)
    ii. Day before surgery and day after
    iii. Before discharge

Regarding fetal echocardiographic evaluation on CDH fetuses, we believe that identification of heart malformations should be the main objective. Suggested parameters of prognostic, like LV hypoplasia, are very demanding, not consensual and without benefit in comparison to recently defined parameters like lung-to-head ratio or lung dimensions achieved by fetal resonance.

After birth, the pediatric cardiologist might be strongly committed in management of CDH patients. In the first hours of life, echocardiography should be performed to assess the presence of congenital heart disease but even in the absence of heart malformation the pediatric cardiologist should be part of the multidisciplinary team and participate in clinical decisions. Pulmonary pressure could be estimated by echocardiography at this time, but generally CDH infants have normal to slightly elevated pulmonary pressure in the first 24 hours of life.

The crucial cardiac assessment of CDH infants should be done at 24 hours of life, with plasmatic NT-proBNP measurement and echocardiography. NT-proBNP seems to have prognostic interest, and levels higher than 11 500 pg/ml is suggestive of worse prognosis and poor survival. Echocardiography should include pulmonary pressure estimation and evaluation of indirect signs of PH severity (ventricular septum and shunt direction). Echocardiographic evaluation should also include parameters of systolic and diastolic bi-ventricular function. If possible, it should be performed by the same observer, in order to exclude inter-observer variation.

The assessment of plasmatic NT-proBNP as well as echocardiographic estimation of pulmonary pressure and heart function might be repeated in case of clinical instability, before and after surgery, and at discharge. Patients with persistent high pulmonary pressure at discharge should be referred to a pediatric cardiology consultation.

Plasmatic NT-proBNP is more useful when evaluated repeatedly and compared to previous results. It is a very sensitive marker, and frequently we found significant increases before clinical deterioration occurs. Other biochemical markers of PH are not proved to be useful in newborns and should not be employed to adjust therapeutics.

Future Directions

At this time several question remains to be defined and would be the key of our future investigation in CDH.

1. We are assessing the clinical expression of several biochemical markers, defined mainly in adults with PH, like uric acid, troponins and ADMA, in order to evaluate its helpfulness as prognostic parameters and monitorization of PH and heart dysfunction.
2. We will investigate the relevance of tissue Doppler in CDH infants, since tissue Doppler is a remarkably method of evaluation of pulmonary pressure and heart function, independent of load conditions.

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